THE UNIVERSITY *of* EDINBURGH

Dissection of the PI3K/Akt/mTOR pathway identifies potential therapeutic targets in canine tumours

Yu-Ting Chen

Thesis presented for the degree of Doctor of Philosophy

The University of Edinburgh

2013

ABSTRACT

Introduction: Over the past decades, considerable advances in understanding of cell biology at genetic, epigenetic and proteomic levels led to development of new strategies for better outcome of cancer therapy. One of these new strategies is targeting the class I PI3K/Akt/mTOR signaling pathway, in that this pathway plays a key role in regulation of many cellular functions, including proliferation, survival, metabolism, autophagy and motility. Dysregulation of the class I PI3K/Akt/mTOR pathway has been documented in a variety of human tumours and inhibition of this pathway has been observed to hamper tumour proliferation *in vitro* and prevent tumour progression *in vivo* and in clinic. More recently, emerging evidence suggests that the class I PI3K/Akt/mTOR pathway is associated with Cancer Stem Cell (CSC) biology, in light of maintenance, viability and conventional therapy resistance of CSCs. The CSC theory conceptualizes that a subset of tumour cells with Stem Cell-like properties, including self-renewal, multipotency, differentiation, and resistance to chemotherapy and radiotherapy, can recapitulate new tumours and resistance to cancer therapy.

Materials and Methods: To explore class I PI3K/Akt/mTOR signaling pathway and CSCs as therapeutic targets in canine oncology, in one series of experiments, small-molecular inhibitors Wortmannin, ZSTK474, KP372-1 and Rapamycin, which selectively target pan-class I PI3K, pan-class I PI3K, Akt and mTOR, respectively, were utilized to treat canine cancer cell lines using inhibitors alone or in combination with conventional therapeutic drugs. The human acute lymphoblastic leukaemia of T-cell origin cell line (Jurkat T cell line) was used as a comparative control. In another, a stem cell culture system was performed to isolate CSCs from canine glioma J3T cell line. Subsequently, microarray analysis of transcriptional expression profiles of J3T spheres (the putative CSCs) versus J3T parental cells was performed.

Results: In this study, small molecules ZSTK474 and KP372-1 were found to significantly decrease cell viability at lower micromolar and nanomolar ranges, respectively. Rapamycin decreased cell viability at lower micromolar concentrations.

However, the efficacy of Wortmannin varied from one cell line to another. Dissection of the mechanism of these inhibitors using Western Blot analysis and annexin V staining showed that all inhibitors functioned by decreasing phosphorylation of class I PI3K pathway members. Notably, the efficacy of Wortmannin for this pathway inhibition is confined to certain cell lines. In addition, Wortmannin had shorter drug duration than the other three inhibitors. Annexin V staining showed that KP372-1 was a potent inducer of apoptosis, with decreasing potency in hierarchy order, Rapamycin, Wortmannin and ZSTK474. The data obtained from the combination of pan-class I PI3K inhibitor (Wortmannin or ZSTK474) and mTOR inhibitor (Rapamycin) suggested that additive/synergistic effects were, in part, due to inactivation of Akt. The class I PI3K pathway inhibitors enhanced the efficacy of Doxorubicin in SB cells but not in canine REM, 3132 and J3T cells.

The CSC colonies of canine glioma J3T cells were successfully isolated and expanded in the neurosphere formation assay. By microarray analysis, several class I PI3K signaling network-associated genes, particularly IGFBP2 (27-fold), FYN (9.3-fold), and DDIT4 (8.5-fold), were found to be highly up-regulated in the J3T CSCs. However, the genes encoding components, such as Akt1 and eIF4E, of class I PI3K/Akt/mTOR axis signaling were either unchanged or down-regulated in the CSCs. The majority of the genes encoding translation initiation factors were also down-regulated in the CSCs.

Conclusions: This study demonstrates that class I PI3K/Akt/mTOR signaling pathway is critical for proliferation and survival of cell lines derived from human acute lymphoblastic leukemia of T cell origin (Jurkat T cell line) and a variety of canine tumours. However, it appears that this pathway is dispensible for maintainence and viability of the CSCs isolated from canine gloma J3T cell line. This study suggests that the strategy of dual inhibition of class I PI3K and mTOR kinases may have better outcomes than the combination inhibitors of this pathway (such as ZSTK474 and KP372-1) with Doxorubicin in canine oncology.

Table of Contents

List of Figures

List of Tables

DECLARATION

This work reported in this thesis was carried out under the supervision of Professor David Argyle, Laboratory for Comparative Oncology and Stem Cell Programme at the Royal (Dick) School of Veterinary Studies and Roslin Institute, The University of Edinburgh. All results presented, unless otherwise stated, are the main work of this author, as is the composition of the thesis.

Yu-Ting Chen Aug 2012

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Professor David Argyle for his support, encouragement and guidance throughout this project.

I am very grateful to Karen A. Tan for her suggestion and support of my work.

I would also like to acknowledge Thalia Blacking, Rhona Muirhead, Lisa Pang, Matylda Sczaniecka, Daniela Gattegno, Jay Yang, Timothy Connelly, Yi-Hsia Liu, Adam Gow, Hannah Geere, Gura Bergkvist, Margaret Ross, Karen Gladstone, Maura Wallace, Sally Argyle for their advice and technical assistance of my work.

Finally, I want to thank my family members, my good friends Orchid Liu, Dorothy Tse, Nicky Craig, Zhen Zhong, Yi-Jing Chen, and Yu-Ching Chang for their endless encouragement and comfort throughout my time in the UK.

Lists of abbreviations

4E-BP1	eukaryotic translation initiation factor 4E (eIF4E) binding protein 1
5’TOP	5' terminal oligopolypyrimidine
5’UTR	5’ untranslated region
A	absorbance
ABC transporter	ATP-binding cassette (ABC) transporter
Abl	Abelson
ACL	ATP citrate lyase
ADA-SCID	adenosine deaminase-deficient severe combined immunodeficiency
ADAP	Adhesion and degranulation-promoting adapter protein
ADP	adenosine diphosphate
AGC kinase family	A protein kinase group is named after cAMP-dependent protein kinase 1 (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C (PKC)
Akt or PKB	protein kinase B
ALDH1	aldehyde dehydrogenase 1
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMPK	AMP-dependent protein kinase
AP-1	activator protein-1
APC	adenomatous polyposis coli
API-2	Akt signaling inhibitor-2
Arf6	adenosine diphosphate (ADP)-ribosylation factor 6
aRNA	antisense RNA
As_2O_3	arsenic trioxide
AS160	Akt substrate of 160 kDa
ASIC2	acid-sensing ion channel-2
Atg13	autophagy-related 13
ATM	ataxia telangiectasia mutated
ATP	adenosine-5'-triphosphate
Bad	B-cell lymphoma-2 (Bcl-2) antagonist of cell death
Bak	Bcl2-antagonist/killer 1
Bax	Bcl2-associated X protein

Bcl-2	B-cell lymphoma-2
Bcl-6	B-cell lymphoma-6
Bcl-X	B-cell lymphoma-related gene
Bcl-XL	B-cell lymphoma-extra large
BCR-ABL	Breakpoint cluster region-Abelson
BCRP	breast cancer resistance protein
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BH	breakpoint-cluster-region homology
Bim	Bcl-2-like protein 11
Bim_{EL}	EL isoform of Bcl-2-like protein 11
BMP	bone morphogenetic protein
Bmx	Bone marrow tyrosine kinase gene in chromosome X protein
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
C/EBPβ	CCAAT/enhancer binding protein-β
CAMs	cell adhesion molecules
CaM	calmodulin
CaMKKβ	calcium/calmodulin-dependent protein kinase kinase 2, beta
CBC	complete blood count
CD64	cluster of differentiation 64
Cdc2	cell division cycle 2
Cdc42	cell division control protein 42 homolog
cDNA	complementary DNA
Chk1	checkpoint kinase 1
CK2	casein kinase 2
CLP	common lymphoid progenitor
CML	chronic myelogenous leukemia
CMP	common myeloid progenitor
CNS	central nervous system
CQ	chloroquine
CREB	cAMP response element-binding
CRP	C-reactive protein
CSC	cancer stem cell
CSH2	C-terminal Src homology 2
CSR	cAMP-stabilizing region
CT	computed tomography
CTMP	carboxyl-terminal modulator protein
DAG	diacylglycerol

DCs	dendritic cells
DAPP1	dual adaptor for phosphotyrosine and 3-phosphoinositides
DDR1	discoidin domain receptor-1
DEG/ENaC	degenerin/epithelial sodium channel
DLL1	Delta-like protein 1
DMEM	Dulbecco’s modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dTMP	thymidine monophosphate
EAAs	essential amino acids
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor 2
eEF2K	eukaryotic elongation factor 2 kinase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	EGFR variant III
eIF	eukaryotic initiation factor
eIF1	eukaryotic translation initiation factor 1
eIF2α	the α subunit of eIF2
eIF2β	the β subunit of eIF2
eIF2γ	the γ subunit of eIF2
eIF3a	the a subunit of eIF3
eIF3e	the e subunit of eIF3
eIF3g	the g subunit of eIF3
eIF3h	the h subunit of eIF3
eIF3i	the i subunit of eIF3
eIF3j	the j subunit of eIF3
eIF4AI	eIF4A isoform 1
eIF4AIII	eIF4A isoform 3
eIF4B	eukaryotic translation initiation factor 4B
eIF4E	eukaryotic translation initiation factor 4E
EMEA	the European Medicines Agency
EMT	epithelial-mesenchymal transition
ENG	endoglin
eNOS	nitric oxide synthase 3 in endothelial cell
ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinase

ES cell	Embryonic stem cell
FasL	Fas ligand
FAT	FRAP, ATM and TRRAP domain
FAT-C	FRAP, ATM and TRRAP (FAT)-C domain
FBP17	formin binding protein 17
FBS	fetal bovine serum
FDA	the U.S. Food and Drug Administration
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGFR1	fibroblast growth factor receptor 1
FGFR2	fibroblast growth factor receptor 2
FIP200	focal adhesion kinase (FAK) family interacting protein of 200 kDa
FISH	fluorescence in situ hybridization
FKBP	FK506 binding protein
FKBP12	FK506-binding protein 12
FKBP38	FK506-binding protein 38
FLNA	Filamin A
Flt1	Fms-like tyrosine kinase 1
Flt3	Fms-like tyrosine kinase 3
Fox	Forkhead box
FoxO1a	Forkhead box O1a
FoxO3a	Forkhead box O3a
FoxO4	Forkhead box O4
FRB	FKBP12-rapamycin binding
g or rcf	relative centrifugal force (rotation speed)
G0 phase	Gap 0 phase (cell cycle)
G1 phase	Gap 1 phase (cell cycle)
G6Pase	glucose-6-phoshatase
Gab1	Grb2-associated binding protein 1
GAP	GTPase-activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gβγ	β and γ subunits of guanine nucleotide binding protein (G protein)
GBM	glioblastoma multiforme
GCOS	GeneChip® Operating Software
GDI	guanosine diphosphate (GDP) dissociation inhibitor
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors

GFAP	glial fibrillary acidic protein
GHR	growth hormone receptor
GISTs	gastrointestinal stromal tumors
GLUT1	glucose transporter type 1
GLUT4	glucose transporter type 4
gp130	glycoprotein 130
GPCR	G protein-coupled receptor
Grb2	growth factor receptor-bound protein 2
GRK2	G protein–coupled receptor kinase 2
GSCs	glioma stem cells
GSK-3α	glycogen synthase kinase 3α
GSK-3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HEAT	huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1
HEK	human embryonic kidney
HES	hairy and enhancer of split
HEY	hairy/enhancer-of-split-related with YRPW-like Motif
HIF	hypoxia-inducible factor
HIF-1α	hypoxia-inducible factors-1α
HDM2	murine double minute 2 (MDM2) in human
HM	hydrophobic motif
HMLEs	human mammary epithelial cells
HNSCC	head and neck squamous cell carcinomas
HRP	horseradish peroxidase
HRs	homology regions
HSCs	hematopoietic stem cells
HSP90	heat shock protein 90 kDa
hTERT	human telomerase reverse transcriptase
hVps34	human vacuolar protein sorting 34
IC-100	inhibitory concentration of 100% cell viability
IFN- γ	interferon-γ
IGF	insulin-like growth factor
IGF-1	insulin-like growth factor-1
IGF-1R	IGF-1 receptor
IGFBP	IGF-binding protein
IκB	inhibitor of the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB)
IκBα	inhibitor of the nuclear factor of kappa light polypeptide gene

	enhancer in B-cells (NF-κB), alpha
IKK-α	inhibitor of nuclear factor kappa-B kinase, alpha subunit
IL-2	interleukin-2
IPA	ingenuity pathway analysis
IRESs	internal ribosome entry sites
IR	insulin receptor
IR-A	Insulin receptor, A isoform
IR-B	Insulin receptor, B isoform
IRS-1	insulin receptor substrate-1
IRS-2	insulin receptor substrate-2
iSH2	inter- Src homology 2
Itk	IL2-inducible T-cell kinase
Jak	Janus kinase
Jak2	Janus kinase 2
JNK	Jun N-terminal kinase
kg	kilogram
LC8	dynein light chain 8 kDa
LCA	Leber congenital amaurosis
LC3B	isoform B of human microtubule-associated protein 1 light chain 3
Lck	lymphocyte-specific protein tyrosine kinase
Lfa-1	lymphocyte function-associated antigen 1
LKB1	liver kinase B1
LOH	loss of heterozygosity
LPC	lysophosphatidylcholines
M	molar
M phase	Mitosis phase (cell cycle)
Mad1	MAX dimerization protein 1
mA	milliampere
MAGI	membrane associated guanylate kinase inverted
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3
MAPK	mitogen-activated protein kinase
MCL	mantle cell lymphoma
Mcl-1	myeloid cell leukemia sequence 1
MDM2	murine double minute 2
MEFs	mouse embryonic fibroblasts
Mek	mitogen-activated protein kinase
Met (M)	Methionine
Met-tRNAi^{Met}	initiator-methionyl-transfer-RNA

µg	microgram
mg	milligram
ml	milliliter
mins	minutes
MK2	MAPK-activated protein kinase-2
MLCK	myosin light chain kinase
mLST8	mammalian lethal with sEC13 protein 8 homolog in *S. cerevisiae*
µM	micromolar
mM	millimolar
MMPs	matrix metalloproteases
MMP2	matrix metalloprotease 2
MMP9	matrix metalloprotease 9
Mnk	MAPK-interacting kinase
MPD	myeloproliferative disorder
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRP-1	multidrug resistance protein-1
mSin1	mammalian stress-activated map kinase-interacting protein 1
MTD	maximum tolerated dose
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
Myt1	myelin transcription factor 1
NCAM	neural cell adhesion molecule
N-CoR	nuclear receptor corepressor
NEAA	non-essential amino acid mix
NEDD4	neural precursor cell expressed, developmentally down-regulated 4
NFAT	nuclear factor of activated T cells
NFAT3	nuclear factor of activated T cells 3
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NGF	nerve growth factor
NICD	Notch intracellular domain
NK	natural killer
nM	nanomolar
nm	nanometer
NO	nitric oxide

NOS2	nitric oxide synthase-2
Notch	neurogenic locus notch homologue protein
NP40	Nonidet-P40
NSC	neural stem cell
NSCLC	non-small cell lung cancer
NSE	neuron-specific enolase
NSH2	N-terminal Src homology 2
NT3	neurotrophin 3
NT4	neurotrophin 4
NT5	neurotrophin 5
ODC	ornithine decarboxylase
p-	phospho-
p120 RasGAP	RAS p21 protein activator 1
$p21^{WAF1}$	cyclin-dependent kinase inhibitor 1A
$p27^{KIP1}$	cyclin-dependent kinase inhibitor 1B
p38MAPK	p38 mitogen-activated protein kinase
p70S6K or S6K1	70 kDa ribosomal S6 kinase
PABP1	poly(A) binding protein, cytoplasmic 1
PAIP-1	poly(A)-binding protein-interacting protein 1
PAIP-2	poly(A) binding protein interacting protein 2
PBS	phosphate buffered saline
PBST	1x PBS containing 0.1% Tween-20
PCR	polymerase chain reaction
PDCD4	programmed cell death 4
PDE3a	phosphodiesterase 3a
PDGF	platelet-derived growth factor
PDGF-B	platelet derived growth factor B
PDGF-C	platelet derived growth factor C
PDGFR	PDGF receptor
PDGFRα	PDGF receptor α
PDGFR β	PDGF receptor β
PDK1	phosphatidylinositol 3-dependent kinase 1
PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2
PFS	progression-free survival
PGC-1	peroxisome proliferator- activated receptor γ coactivator-1
PH	pleckstrin homology
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PI4K	phosphatidylinositol 4-kinase

PIKK	phosphinositide-3-kinase-related kinase
PIM	proviral integration sites associated with murine leukemia virus –induced lymphomagenesis
Pink1	PTEN-induced kinase 1
PKA	cAMP-dependent protein kinase 1
PKC	protein kinase C
PKCι	protein kinase C iota
PKCδ	protein kinase C delta
PKD	protein kinase D
PKG	cGMP-dependent protein kinase
PKN	PKC-related protein kinase
PLC-γ	phospholipase C, gamma type
PLC-ε	phospholipase C, epsilon type
PLD	phospholipase D
PLD3	phospholipase D3
PML	promyelocytic leukaemia protein
pNET	pancreatic neuroendocrine tumours
PP1	protein phosphatase 1
PP1γ	the catalytic subunit of protein phosphatase 1 (PP1), γ isoform
PP2A	protein phosphatase 2A
PP2Ac-β	protein phosphatase 2, catalytic subunit, beta isoform
PP2C-ζ	protein phosphatase 2C, zeta isoform
PPARγ	peroxisome proliferator-activated receptor γ
PRAS40	proline-rich Akt/PKB substrate 40 kDa
Protor-1	protein observed with Rictor 1
PS	phosphatidylserine
Ptch1	Patched 1
PtdIns(4,5)P_2 or PIP2	phosphatidylinositol-4,5–biphosphate
PtdIns(3,4,5)P_3 or PIP3	phosphatidylinositol-3,4,5–triphosphate
PTEN	phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf	v-raf murine sarcoma/leukemia viral oncogene homolog
Rag	Ras-related small GTP-binding protein
Raptor	regulatory-associated protein of mTOR complex 1
RCC	renal cell carcinomas
REDD1	regulated in development and DNA damage 1
pRb	retinoblastoma protein

Rheb	Ras homolog enriched in brain
Rho	ras homolog gene family
RhoC	ras homolog gene family (Rho), member C
RhoJ	ras homolog gene family (Rho), member J
RhoQ	ras homolog gene family (Rho), member Q
RhoT1	ras homolog gene family (Rho), member T1
Rictor	rapamycin-insensitive companion of mTOR
RMA	Robust Multichip Averaging
Rnd3	Rho family GTPase 3
ROSs	reactive oxygen species
RRAS	ras-related protein
RSK	90 kDa ribosomal protein S6 kinase
RSK1	90 kDa ribosomal protein S6 kinase (RSK), isoform 1
RTK	receptor tyrosine kinase
RyRs	ryanodine receptors
S phase	DNA synthesis phase (cell cycle)
S6RP	S6 ribosomal protein
SC	stem cell
SCID mouse	severe combined immunodeficiency disease mouse
SDs	standard deviations
SDF-1	stromal cell-derived factor-1
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser (S)	Serine
SGK	serum- and glucocorticoid-induced protein kinase
SH2	Src homology 2
SH3	Src homology 3
Shh	sonic hedgehog
Shp-2	SH2 domain-containing tyrosine phosphatase 2
shRNA	short hairpin RNA
siRNA	small interfering RNA
SC	stem cell
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SKAP	Src kinase-associated phosphoprotein
SKAR	46 kDa DNA polymerase delta interaction protein
SLC1A5	solute carrier family 1 member 5
SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+

	system), member 5
SLE	systemic lupus erythematosus
SM	systemic mastocytosis
Smo receptor	Smoothened receptor
SNPs	single nucleotide polymorphisms
SOCS	suppressor of cytokine signaling
SP	side population
SREBP	sterol regulatory element binding protein
SRF	serum response factor
SSCP	single strand conformation polymorphism
Stat	signal transducers and activators of transcription
Stat3	signal transducers and activators of transcription 3
SUCCEED	Sarcoma mUlti-Center Clinical Evaluation of the Efficacy of riDaforolimus
TCR	T cell antigen receptor
Tec	tyrosine-protein kinase
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFβ	transforming growth factor beta
TβRI	transforming growth factor beta (TGFβ) receptor type I
TβRII	transforming growth factor beta (TGFβ) receptor type II
Thr	threonine
TKIs	tyrosine kinase inhibitors
TM	turn motif
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-alpha
TRADD	tumor necrosis factor receptor-associated death domain
TRAIL	tumor necrosis factor (TNF)-related apoptosis inducing ligand
Trip10	thyroid hormone receptor interactor 10
TSC1	tuberous sclerosis complex 1
TSC2	tuberous sclerosis complex 2
Twist-1	twist homolog 1 in *Drosophila*
Tyr (Y)	tyrosine
U	unit
ULK1	Unc-51 like kinase 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VEGFR2	vascular endothelial growth factor receptor 2
VSM	vascular smooth muscle

v/v	volume to volume
Wee1Hu	Wee1 homolog in *S. pombe*
WM	Waldenström's macroglobulinemia

Chapter 1: Introduction

1.1 Cancer

Cancer is a disease, which is characterized by uncontrolled proliferation of a subset of cells. In a survey of fifty countries in 2008, about 202-326 cancer cases per 100,000 people were reported (Bray F *et al.* 2010; Ferlay J *et al.* 2010). The etiology of cancer is a subset of cells with molecular alterations at genetic level, along with aberrant post-translational modifications, as a result of a variety of factors. These factors that drive cell transformation encompass chemical and physical carcinogens, hormone factors, infections by microorganism (e.g. virus and bacterium) and heredity factors (Hisada *et al.* 1998; IARC-Working-group 2000; Kuper *et al.* 2000; Konduri and Schwarz 2007). Once the normal cells are transformed to the cancer cells, they undergo unlimited cell division, uncontrolled growth and escape programmed cell death. When the cancerous cells become more malignant, they start to invade surrounding tissue and even metastasize to distant organs/tissues via lymphatic and hematological systems. In addition, many malignant cancer types produce paraneoplastic syndromes, such as cachexia, hypercalcemia and hypoglycemia, in human and canine cancer patients. As cancer progresses, it frequently disturbs or destroys normal functions of tissues/organs and cause cachexia and death at the terminal stage (Morrison 1979; Finora 2003; Withrow and Vail 2007; Pelosof and Gerber 2010).

1.1.1 The hallmarks of cancer

At molecular level, cancer cells are characterized by six crucial traits, which make the disease more malignant. These six traits are self-sustenance of growth signals, unresponsiveness to growth inhibitory signals, escape of apoptosis, infinite DNA replication, angiogenesis, and invasive and metastatic behaviors (Hanahan and Weinberg 2000). Cancer cells are notorious for not being regulated by exogenous growth factor stimulation. Instead, they develop their own systems to constitutively activate growth signaling due to the occurrence of molecular alterations of components of one or more growth signaling pathway(s). For example, activating mutations in receptor tyrosine

kinases (RTKs), the growth factor-binding receptors, have been found to cause constitutive activation of growth signaling pathways, in the absence of exogenous growth factors (Hirota *et al.* 1998; Meshinchi and Appelbaum 2009). Moreover, some cancer cell types such as human osteosarcoma and glioma have been reported to produce growth factors on their own, thus maintaing active growth signaling pathways (Sporn and Roberts 1985).

Besides self-sustenance of growth signals, cancer cells grow in an uncontrolled manner, in part, by acquiring the ability of unresponsiveness to growth inhibitory signals such as transforming growth factor β (TGFβ). Colon cancer cells with decreased expression of TGFβ receptor are reported to be less sensitive to the stimulation of TGFβ signal, thus allowing colon cancer cells to evade growth inhibition regulation (Markowitz *et al.* 1995). It should be mentioned that TGFβ signaling and many other growth inhibitory pathways have been found to converge on retinoblastoma protein (pRb), which is the first identified tumour suppressor and acts to arrest G1/S phase trasition (Hanahan and Weinberg 2000). Decreased or loss of pRb activity, as a result of either pRb gene mutation or degradation of pRb protein by DNA viruses such as human papillomavirus and simian virus 40, have been observed in a subset of human tumours, including retinoblastoma, small cell lung carcinoma and bladder carcinoma (DeCaprio *et al.* 1988; Munger *et al.* 1989; Horowitz *et al.* 1990; Classon and Harlow 2002). Loss of pRb function is generally condisdered to accelerate tumour transformation (Horowitz *et al.* 1990; Classon and Harlow 2002).

The third trait of cancer cells is to acquire the ability of escaping programmed cell death. To date, many molecular alterations of oncogenes and tumour suppressor genes, which are involved in apoptotic machinery, have been identified. p53, as one of the early identified tumour suppressors, is known as a pro-apoptosis factor and participates in DNA repair and cell cycle control (Harris 1996). Mutations of p53 gene, which frequently occur in a variety of human cancer types, can result in down-regulating p53-regulated cellular activities and inducing tumourigenesis through a wide array of p53-targeted genes (Lane and Benchimol 1990; Dittmer *et al.* 1993; Greenblatt *et al.*

1994). Molecular alterations of the pro-survival factor Akt often result in promoting the growth, survival and invasive behavior of cancer cells (Altomare and Testa 2005).

Unlike the occurrence of senescence in normal cells, cancer cells are found to maintain immortality through several mechanisms. One of the mechanisms to protect cancer cells from senescence is to up-regulate telomerase, which acts to add DNA sequence repeats TTAGGG onto the telomere regions located at the both ends of chromosomes, resulting in cancer cells acquiring the ability of infinite DNA replication. In normal cells, the length of telomere is gradually shoterned with each time during DNA replication (S phase), which often leads to end-to-end fusion of two chromosomes with loss of telomeres and consequently initiating cell apoptosis (Counter *et al.* 1992; Shay *et al.* 2001).

Since 1971, Judah Folkman has discovered angiogenesis, a process of the budding of new capillaries from pre-existing blood vessels, as a common phenomenon during tumour expansion (Folkman 1971). The critical role of angiogenesis in the supply of nutrients and oxygen for tumour development is further supported by a body of literature (Kim *et al.* 1993; Hanahan and Folkman 1996; Arbab 2012; Kubota 2012). To date, several pro-angiogenesis factors such as vascular endothelial growth factor (VEGF), VEGF receptors (VEGFRs), basic fibroblast growth factor (bFGF) and angiopoietins have been identified to be released from cancer cells (Murakami and Simons 2008; Takahashi 2011). Moreover, anti-angiogenesis agents targeting VEGF or VEGFRs have been either investigated in clinical trials of malignant gliomas or approved for clinical use, in combination with chemotherapy, to treat metastatic colon cancers (Los *et al.* 2007; Dietrich *et al.* 2008).

As cancer progresses, a subset of cancer cells invade adjacent tissues, and even, metastasize to distant organs and tissues via blood stream and lymph, leading to expanding tumour size and founding new colonies. Cancer cells with invasive and/or metastatic phenotypes often utilize two main mechanisms for cancer cell spreading (Hanahan and Weinberg 2000). The first mechanism is to alter expression of cell

adhesion molecules (CAMs) such as E-cadherin, neural cell adhesion molecule (NCAM) and integrins. Down-regulation of E-cadherin, conversion of highly adhesive NCAM to loss of adhesive function of NCAM, and up-regulation of certain integrins such as α3β1 that favors cell migration have been reported to promote invasion and metastasis of cancer cells (Kaiser *et al.* 1996; Christofori and Semb 1999; Gogineni *et al.* 2011). The second mechanism is to up-regulation of extracellular matrix (ECM)-degrading proteases such as matrix metalloproteinases (MMPs) (Hanahan and Weinberg 2000). Interestingly, despite some MMPs being produced by cancer cells, the majority of these proteins are released from stromal cells within and surrounding tumour mass (Egeblad and Werb 2002). Proteolysis of ECM by MMPs not only allows cancer cells to invade the adjacent tissue but also favors the proliferation and migration of endothelial cells for angiogenesis (Stetler-Stevenson 1999).

To date, mounting evidence has suggested that accumulation of genetic alterations not only causes the onset of cancer but also contributes to cancer development (Kinzler and Vogelstein 1996; Bailey and Murnane 2006). In a study of heredity colorectal cancer, at least 5 genetic alterations have been considered as the lowest threshold of giving rise to a malignant tumour (Kinzler and Vogelstein 1996). Later, another study of sporadic (nonhereditary) colorectal cancer has found that each carcinoma cell contains around 11,000 events of genetic alterations (Stoler *et al.* 1999). In cancers, there are four types of genetic alterations, including few base pair mutations, changes in chromosome numbers, chromosome translocations, and gene amplificaions, the latter three belonging to chromosomal instability (Lengauer *et al.* 1998). Although DNA polymerases and two DNA repair systems (mismatch repair and nucleotide-excision repair) control the rate of mutation accumulation, the majority of mutations are frequently caused by a defect in mismatch repair. In contrast to defective mismatch repair found in few cancer types, chromosome instability is commonly found in most human cancer types (Lengauer *et al.* 1998; Negrini *et al.* 2010). Alterations of genes, which regulate cell cycle checkpoints and mitotic cell division associated with replication and segregation of chromosomes, can result in chromosomal instability

(Lengauer *et al.* 1998). For example, down-regulation of p53 tumour suppressor gene, which serves as a DNA-damage checkpoint gene, is involved in exacerbating chromosomal instability at the later stage (but not the early stage) of colorectal cancer development (Baker *et al.* 1990; Kinzler and Vogelstein 1996). Hyperactivation of mitotic checkpoint, resulting in extension of mitosis phase, lagging chromosomes and subsequent changes in chromosome numbers, have been observed in many human cancers (Schvartzman *et al.* 2010). Telomere dysfunction, as a result of abnormality of telomere-related proteins or sudden loss of telomeric repeats (TTAGGC), can promote the end-to-end fusion of two chromosomes and generate DNA amplifications and translocations (Bailey and Murnane 2006).

1.1.2 Detection and diagnosis of cancer

Most cancers are detected initially through diagnostic imaging tools, such as radiography, computed tomography (CT) and magnetic resonance imaging (MRI). Routine blood tests including complete blood count (CBC) and biochemical serum profile are performed to diagnose hematological malignancy and certain cancer types with paraneoplastic syndromes. Pathological examinations of specimens obtained from tumour mass and its margin, after surgical resection or biopsy, not only give a definite diagnosis of these specimens but also guide clinicians to prescribing a suitable treatment for patients with cancer (Sausville and Longo 2001; Withrow and Vail 2007).

1.1.3 Treatment of cancer

1.1.3.1 Surgery

To cure patients with cancer, conventional therapeutic options are surgery, radiation therapy and chemotherapy. Among the three conventional therapies, surgery is considered to be the first choice for cancer treatment, in that a bulky tumour mass with its margin of normal tissue can be completely removed. Even if complete removal of a tumour is not accessible, partial resection of a tumour mass may help to relieve the

burden of local organs and tissue in a cancer patient or the pain of spinal cord decompression (Sausville and Longo 2001). It has been reported that some patients with metastatic tumours, such as osteosarcoma metastasizing to lung, can survive for 5 years after surgical resection of these metastasized tumours (Pfannschmidt *et al.* 2012). For certain hormone-responsive tumours such as androgen-responsive prostate and estrogen-responsive breast cancers, surgical orchiectomy and oophorectomy/adrenalectomy are performed to decrease production of androgen and estrogen, respectively (Strong 1963).

1.1.3.2 Radiation therapy

With regard to radiation therapy, X-rays and gamma rays are the forms of radiation frequently used for cancer treatment. Whilst X-rays are produced by linear accelerators, gamma rays are emitted from radionuclides, such as cobalt-57 and manganese-54, which undergo radioactive decay (Sausville and Longo 2001). X-rays and gamma rays are able to ionize and excite atoms and molecules in cells, leading to production of ions and oxidative free radicals. These products hit the targeted chromosomes, which in turn causes chromosomal breaks and disturbance of cell division, leading to cellular death. Tumour cells are more sensitive to radiation than normal cells, in that the repair kinetics of chromosomal breaks in tumour cells is slower than that in normal cells (Sausville and Longo 2001; Kawata *et al.* 2004). Radiation can be used as either primary or adjuvant therapy. Primary radiation therapy is employed for the treatment of small malignant solid tumours that are considered to be highly curable by radiation. Primary radiation therapy is also performed in tumours located at the sites where surgery is difficult to be performed or complete resection of a tumour is inaccessible due to anatomic limitation and inadequate skin for wound healing or cosmetic surgery (Theon 2000; Sausville and Longo 2001). For dog and cat patients, primary radiation therapy is suitable for tumours growing on the head, such as carcinomas of the eyelid and nose, and around specific anatomic sites such as spinal cord and large vessels. Adjuvant radiation therapy is frequently performed following surgical resection of gross lesions, for the purpose of prevention of dissemination and

metastasis of tumour cells (Theon 2000). Although radiation can be used to kill tumour cells, it also generates toxicities in human patients including fatigue, anorexia, nausea and vomiting. Hypoxic or resting cells are more resistant to radiation than their normaxic or proliferating counterparts. For instance, neuron cells are found to be highly radioresistant whereas bone marrow is radiosensitive (Pizzarello and Witcofski 1972).

1.1.3.3 Chemotherapy

Chemotherapy is often employed to eradicate tumour cells after surgical resection of gross lesions or to treat metastatic diseases. There are many commercial chemotherapeutic agents avaibable for cancer treatment, which include antimetabolites, purines, pyrimidines, vinca alkaloid, alkylating agents and antitumour antibiotics. Based on the mechanism, these agents can be classified into drugs that directly target deoxyribonucleic acid (DNA), drugs that indirectly affect DNA functions, mitotic spindle inhibitors and hormone agents. Drugs that directly target DNA through formation of covalent bonds are alkylating agents such as Cyclophosphamide and Chlorambucil, and platinum compounds such as Cisplatin and Carboplatin. Some antitumour antibiotics, such as Doxorubicin, Bleomycin and Mitomycin C, can bind to DNA and cause single strand breaks of DNA (Chabner and Longo 2001). For Doxorubicin and its derivatives, their interaction with DNA not only prevents synthesis of DNA and RNA but also stops the activity of topoisomerase II (Momparler *et al.* 1976; Burgess *et al.* 2008). Drugs that indirectly affect DNA functions are antimetabolite family such as Methotraxate, 5-Fluorouracil and Cytosine arabinoside (Chabner and Longo 2001). Methotrexate inhibits the activity of dihydrofolate reductase that up-regulates production of folic acid that is required for synthesis of nucleoside thymidine, a critical DNA base, through conversion of dihydrofolate into tetrahydrofolate (Rajagopalan *et al.* 2002). 5-Fluorouracil is a pyrimidine analogue and functions as an inhibitor of thymidylate synthase that can generate thymidine monophosphate (dTMP) which is required for DNA synthesis (Longley *et al.* 2003). Cytosine arabinoside, an analogue of a DNA base cytosine deoxyribose, induces cell death through incorporating

itself into DNA and inducing S phase-associated toxicity (Braess *et al.* 1999). Vinca alkaloid and taxane families act as inhibitors of mitotic spindle. For instance, Vincristine belongs to a vinca alkaloid family and functions in interaction with α and β tubulins of microtubule. The microtubule is a component of cytoskeleton and plays a role in chromosomal migration during mitosis phase, maintenance of cell shape during interphase and cell motility. The binding of Vincristine to subunits of microtubules causes depolymerization of microtubules, resulting in inhibition of DNA mitosis (Cooper 2000; Sausville and Longo 2001). The taxane family such as Pacitaxel and Docetaxel prevents DNA from completing mitosis by aberrantly stabilizing microtubules (Fata *et al.* 1999; Nagano *et al.* 2007). Hormone agents such as Glucocorticoids and Tamoxifen, have been reported for the treatment of hematopoietic malignancies, breast and prostate cancers, respectively (Osborne 1998; Schmidt *et al.* 2004). Glucocorticoid acts as an inducer of apoptosis in tumour cells through direct modulation of apoptotic or survival genes and indirect modulation of cellular stress genes (Schmidt *et al.* 2004). Tamoxifen, an antagonist of the estrogen receptor, is efficacious for the treatment of breast tumours with overexpression of estrogen receptors (Osborne 1998). Most chemotherapeutic agents that are introduced to human oncology can also be used in dogs and cats. However, Cisplatin may cause pulmonary toxicity, emesis and other potential side effects in cats, whereas its derivative Carboplatin is safe for cats (Knapp *et al.* 1987; Rudd *et al.* 2000). 5-Fluorouracil has neurotoxic effects on dogs and cats (Harvey *et al.* 1977; Dorman *et al.* 1990; Moore and Frimberger 2000).

Although chemotherapeutic agents are cytotoxic to tumour cells, they also cause acute toxicities to the human patients. Myelosuppression, nausea, vomiting, alopecia, gonadal dysfunction and decreased fertility are adverse effects that are commonly seen in cancer patients who receive chemotherapy. Some drugs may cause specific adverse effects. For instance, Doxorubicin often induces cardiac toxicity in humans (Licata *et al.* 2000). In veterinary oncology, most adverse effects induced by chemotherapeutic agents are similar to those observed in human oncology, with the exception of alopecia that is rarely a problem in the dogs and cats that have fur. In addition, Cisplatin is not

recommended for the dogs with pre-existing renal diesease, or with urinary tract tumours, due to the drug-related nephrotoxicities. Like human, Doxorubicin induces cardiotoxicity in the dog breeds that are susceptible to cardiac diseases and the dog patients with cardiovascular diseases (Moore and Frimberger 2000).

1.1.3.4 Immunotherapy

With the considerable advances in application of genomic and proteomic technology to cells over the past decades, more and more information regarding genes that are aberrantly expressed or silencing in cancer cells as well as immune response against tumour are being unraveled. This inspires scientists to develop new apporoches to treat cancer. These new approaches include immunotherapy and molecular/targeted therapy. The immunotherapy is to stimulate either nonspecific or specific immune responses to antagonize cancer progression. For instance, Bacillus Calmette-Guerin (BCG), a vaccine prepared from the attenuated *Microbacterium bovis*, is currently used to treat nonmuscle-invasive bladder cancer, due to BCG-induced attraction and activation of immune cells such as neutrophils, macrophages, and natural killer (NK) cells and generation of cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN- γ) (Patard *et al.* 1998; Lodillinsky *et al.* 2010). Other microorganisms and their products, such as attenuated virulence of *Samonella typhimurium,* oncolytic adenovirus, and *Staphylococcus aureus*-related superantigens, have been developed for immunotherapy against tumours (Ries and Brandts 2004; Chorobik and Marcinkiewicz 2011; Han *et al.* 2011). Delivery of recombinant cytokines such as IL-2 and IFNs into tumour cells to trigger both innate and adaptive immune responeses has been tested in metastatic melanoma (Schadendorf *et al.* 2009). In addition, vaccines aimed to kill cancer cells are under development and efficacy of some of these vaccines has been assessed in clinical trials. These vaccines can be prepared from whole tumour-cell lysates, plasmid DNA that carries a targeted gene encoding immunogen, viral vector carrying genes encoding tumour-associated antigen or cytokines, or antigen-presenting dendritic cells (DCs)

loaded with tumour-associated antigens (Sondak and Sosman 2003; Shaw and Strong 2006; Larocca and Schlom 2011; Onishi *et al.* 2011).

1.1.3.5 Molecular/targeted therapy

About molecular/targeted therapy to treat cancer, early studies focused on development of gene therapy, which was to replace a mutated gene by delivery of a gene of interest into cells through viral or non-viral vector. These targeted genes could encode tumour suppressors such as p53, cytokines such as IL-2, or encode enzymes that can convert a nontoxic prodrug to cytotoxic agent that can drive cancer cells to death (Withrow and Vail 2007). Although clinical trials of gene therapy were initiated since 1990s, this technique encountered the hurdles of safety issue of gene delivery and whether gene could be delivered to targeted cells, instead of miscellaneous tissues and organs. These challenges slowed the progress of gene therapy (Blaese *et al.* 1995; Sheridan 2011). Recently, successful cases of gene therapy for Leber congenital amaurosis (LCA), adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID), X-linked adrenoleukodystrophy, and Parkinson's disease, have been reported (Cartier and Aubourg 2010; Ferrua *et al.* 2010; LeWitt *et al.* 2011; Stein *et al.* 2011). The technical breakthrough of gene therapy encourages its wide application to treatment of a variety of diseases.

In addition to gene therapy, other targeted therapies including RNA interference, small molecular inhibitor, and monoclonal antibody are currently under development and evaluated in preclinical experiments and clinical trials for the treatment of cancer and many other diseases (Silva *et al.* 2008; Kwitkowski *et al.* 2010; Loi *et al.* 2011). In the field of cancer treatment, these targeted therapies are developed to block specific cellular component(s) which are critical for promoting growth, survival and progression of cancer. As cancers go through multiple steps of molecular alterations, which leads to shaping its own signaling network, many cellular components have been identified to participate in cell transformation and cancer progression. For instance, receptor tyrosine kinases (RTKs) are popular targets for oncology therapy because they are mainly

responsible for transducing signals to regulate cellular growth and survival and angiogenesis after RTKs are activated through binding to growth factors (Lemmon and Schlessinger 2010). Telomerase is another example of being a therapeutic target for cancer treatment due to its important role in prolonging life span of cancer cells through maintenance of the length of telomeres at the chromosome ends (Xu *et al.* 2011). Novel therapeutic targets such as aberrant methylation of DNA and histone deacetylation, resulting in epigenetic and transcriptional dysregulation in cancer cells, are being employed in the preclinical and clinical studies of hematological malignancies (Bishton *et al.* 2007).

As accumulating evidence implicated a strong correlation of aberrant activation of class I phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of Rapamycin (mTOR) signaling pathway with the growth and progression of cancer, this pathway has become a popular therapeutic target and most of the functions of this pathway have been well-studied. The class I PI3K/Akt/mTOR signaling pathway has been well-established for its role in regulation of several cellular processes, including proliferation, survival, metabolism, autophagy and motility of cells. Hyperactivity of this pathway has been shown to result in tumourigenesis, angiogenesis and malignancy behavior (Bjornsti and Houghton 2004; Altomare and Testa 2005; Bertelsen *et al.* 2006). Currently, many therapeutics that target the class I PI3K pathway have been developed and investigated in *in vitro* and *in vivo* experiments, and clinical trials for cancer treatment. Some class I PI3K pathway-targeted drugs such as Temsirolimus and Everolimus have been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the treatment of advanced/relapsed renal cell carcinoma (RCC), due to the promising outcomes of administration of the two drugs in clinical settings (Yuan *et al.* 2009). Although not all cancer types responded well to the class I PI3K pathway-targeted monotherapy, strategies for combining inhibitors targeting this pathway with surgery/chemotherapy/radiation therapy/targeted therapies toward other signaling pathways are investigated in ongoing clinical trials or preclinical studies (http://clinicaltrials.gov/ct2/home) (Ciuffreda *et al.* 2010).

Although a large body of literature addressed the important role of class I PI3K/Akt/mTOR pathway in human cancers, there are only few published data from canine oncology. Since 2008, the activity and pharmacological inhibition of the class I PI3K/Akt/mTOR pathway have been investigated in canine melanomas, mastocytomas *in vitro* and canine osteosarcoma *in vitro* and in an ongoing clinical trial (Rebuzzi *et al.* 2007; Gordon *et al.* 2008; Kent *et al.* 2009; Paoloni *et al.* 2010). Rapamycin that targeted mTOR kinase was the only one targeted drug used in these studies. Treatment with Rapamycin resulted in reduced signal transduction of mTOR complex 1 (mTORC1) pathway and proliferation of tumour cells were observed in two out of three melanoma cell lines and all tested osteosarcoma cell lines (Gordon *et al.* 2008; Kent *et al.* 2009; Paoloni *et al.* 2010). In the study of pharmacokinetic and pharmacodynamics of Rapamycin, the doses tested were all well-tolerated in the patients with canine osteosarcomas (Paoloni *et al.* 2010). In the C2 cell line that was derived from canine mastocytoma, Rapamycin significantly decreased mTORC1-associated expression of vascular endothelial growth factor (VEGF) protein but it failed to inhibit proliferation (Rebuzzi *et al.* 2007).

1.2 Class I PI3K/Akt/mTORC1 axis pathway

The class I PI3K signaling pathway is composed of a series of serine (Ser)/threonine (Thr) kinases that can transduce signals through phosphorylation. In the canonical phosphorylation process adenosine-5'-triphosphate (ATP) is positioned at the catalytic domain in a given protein kinase, and the γ-phosphate group is transferred from ATP to serine or threonine residue(s) of a protein substrate, leading to activation or inhibition of the substrate (Johnson *et al.* 1996).

Class I PI3Ks, which are lipid kinases, are further classified into class IA and IB PI3Ks, according to the differences in regulatory subunits and upstream regulators. Class I PI3Ks are heterodimers that are composed of a catalytic subunit and a regulatory subunit.

1.2.1 Upstream activators of class IA PI3K

Class IA PI3K consists of a p110α/p110β/p110γ catalytic subunit and a p85 regulatory subunit whereas Class IB PI3K is composed of a p110γ catalytic subunit and a p101 or p87/p84 regulatory subunit (Wymann and Pirola 1998; Kong and Yamori 2008; Kurig *et al.* 2009; Vanhaesebroeck *et al.* 2010). Class IA PI3Ks are predominantly activated by RTKs upon receiving growth factors. There are three ways for RTK to recruit and activate class IA PI3K. The first way is that the activated RTKs undergo autophosphorylation of tyrosine (Tyr, Y) residues and formation of YXXM motif (where X represents any amino acid and M is an abbreviation for methionine) at the intracellular domains of RTKs. These YXXM motif are soon recognized by cytoplasmic protein kinases which contain Src homology-2 (SH2) domain and one of these SH2-containing proteins is p85 regulatory subunit of class IA PI3Ks. The interaction between YXXM motives of RTK and SH2 domain of p85 allows RTK to recruit class IA PI3K to the inner side of plasma membrane. The second way is that RTK phosphorylate Y residues on its substrates proteins, such as insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2) and Grb2-associated binding protein 1 (Gab1), which results in generating YXXM motives on these substrates and thereby recruiting class IA PI3K. Alternatively, RTK recruits class IA PI3K through Ras. RTK-mediated Ras activation allows Ras to phosphorylate catalytic p110 subunits of class IA PI3K (Figure 1.01) (Rodriguez-Viciana *et al.* 1996; Kurig *et al.* 2009; Vanhaesebroeck *et al.* 2010). In addition to RTKs, some cytokine receptors, ligand-binding can trigger tyrosine kinase Janus kinase 2 (Jak2), which in turn phosphorylates Y residues on IRS-1/IRS-2/IRS-3, thus triggering class IA PI3K activity (Wymann and Pirola 1998; Schlessinger 2000).

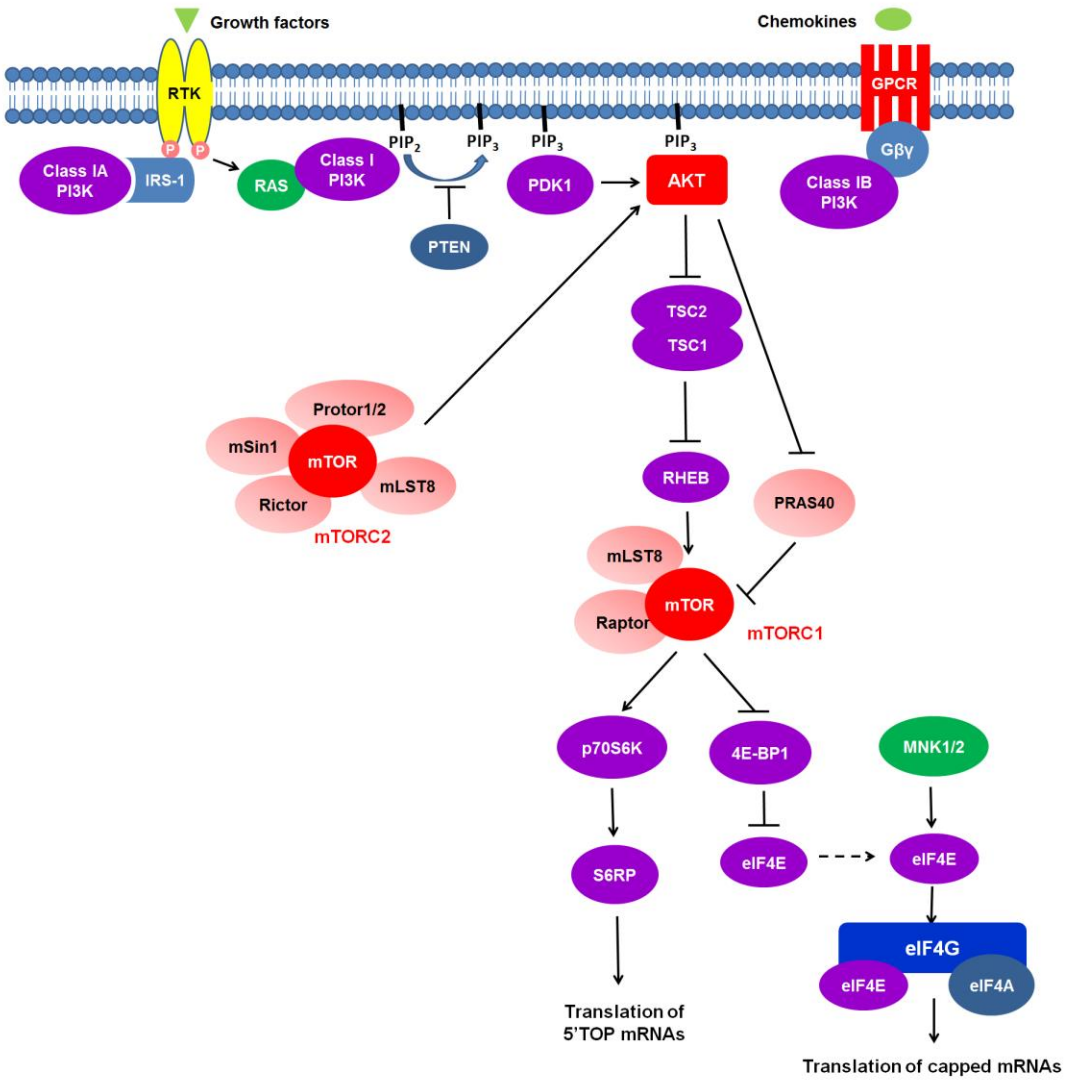


Figure 1.01. Class I PI3K/Akt/mTOR signaling pathway. Arrows represent active phosphorylation. Bars represent inhibitory phosphorylation. PIP2, phosphatidylinositol-4,5–biphosphate; PIP3, phosphatidylinositol-3,4,5–triphosphate. For the full names of the proteins indicated in this figure, see **Lists of Abbreviations**.

1.2.2 Upstream activators of class IB PI3K

Class IB PI3K is activated by G protein-coupled receptors (GPCRs) after chemokine and transmitter stimulation. The activated GPCRs initially bind to βγ subunits of guanine nucleotide binding protein (G protein) (Gβγ), which in turn allows Gβγ to recruit p101 or p87/p84 regulatory subunits of class IB PI3K and activate class IB PI3K (Engelman *et al.* 2006). Alternatively, GPCRs can indirectly activate class IB PI3K through Ras. After Ras is activated by GPCRs, the Ras can phosphorylate p110γ and subsequently activate class IB PI3K (Figure 1.01) (Wymann and Pirola 1998; Schlessinger 2000).

1.2.3 Activation of class I PI3K-dependent Akt/mTOR axis pathway

The activated class IA and IB PI3Ks transfer the γ-phosphate group of ATP to phosphatidylinositol-4,5–biphosphate (PtdIns(4,5)P_2), leading to the conversion of PtdIns(4,5)P_2 to phosphatidylinositol-3,4,5–triphosphate (PtdIns(3,4,5)P_3). The PtdIns(3,4,5)P_3 can recruit phosphatidylinositol 3-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt, hereafter referred to as Akt) to the inner side of cell membrane. PDK1 has been reported to be constitutively activated in cytosol due to its auto-phosphorylation on serine (Ser)241 and its activity can be up-regulated up to ~1000 fold following PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3-mediated cytoplasmic membrane localization (Alessi *et al.* 1997; Casamayor *et al.* 1999). After PDK1 is translocated to cell membrane, it can phosphorylate Akt on Ser308, which lead to Akt activation and triggering a series of events, including promotion of cell survival, growth and protein synthesis, all of which are under the control of Akt (Figure 1.01) (Wymann and Pirola 1998; Lemmon and Schlessinger 2010; Pearce *et al.* 2010).

Signal transduction by class I PI3K kinase cascades can be inhibited or reversed by phosphatase and tensin homolog (PTEN) tumour suppressor catalyzing cleavage of the phosphate group at position 3 on PtdIns(3,4,5)P_3. As a result, PtdIns(3,4,5)P_3 is

converted back to PtdIns(4,5)P_2, thus preventing PDK1 and Akt from being recruited to plasma membrane (Figure 1.01) (Maehama and Dixon 1998).

Once Akt is activated, it can transduce signals to mTORC1 and activate mTORC1 pathway. There are two ways for Akt to activate mTORC1. The first way is that Akt directly phosphorylates two residues, Ser939 and threonine (Thr) 1462, on tuberous sclerosis complex 2 (TSC2) (Manning *et al.* 2002). The de-phosphorylated TSC2 is in complex with tuberous sclerosis complex 1 (TSC1) to form a TSC1–TSC2 complex heterodimer. The TSC1–TSC2 complex has GTPase-activating protein (GAP) activity towards Rheb (for Ras homolog enriched in brain) a member of the Ras superfamily of small G proteins. The TSC1-TSC2 complex-mediated GAP activity causes a reduction in guanosine triphosphate (GTP)-bound Rheb and an increase in guanosine diphosphate (GDP)-bound Rheb. The Akt-dependent phosphorylated TSC2 suppresses the GAP-activity of the TSC1-TSC2 heterodimer, allowing Rheb to remain in the active, GTP-bound state (Manning and Cantley 2003). The active GTP-bound Rheb acts as a positive regulator of mTORC1 through direct interaction with the catalytic (or kinase) domain in mTOR. In addition to mTOR, GTP-bound Rheb has two additional targets, FK506-binding protein 38 (FKBP38) and PLD1, both of which have been considered to be possible candidates for mTOR activation. However, further investigation into the roles of these two proteins is required (Avruch *et al.* 2009). Alternatively, Akt phosphorylate proline-rich Akt/PKB substrate 40 kDa (PRAS40), which impairs PRAS40-mediated inhibition of mTORC1 activity (Figure 1.01) (Sancak *et al.* 2007; Vander Haar *et al.* 2007). Although an early study suggested that mTORC1 acted as a direct substrate of Akt through phosphorylation of Ser2448 on mTOR, a recent study re-evaluated this phosphorylation site and clarified that 70kDa ribosomal S6 kinase (p70S6K or S6K1) was the predominant positive regulator (Scott *et al.* 1998; Holz and Blenis 2005).

mTORC1 is composed of mTOR, regulatory-associated protein of mTOR complex 1 (Raptor) which is uniquely present in mTORC1, proline-rich Akt/PKB substrate 40 kDa (PRAS40) which acts as an mTORC1 negative regulator, and

mammalian lethal with sEC13 protein 8 homolog in *S. cerevisiae* (mLST8) (Figure 1.01) (Sancak *et al.* 2007; Vander Haar *et al.* 2007; Ramirez-Valle *et al.* 2010). mTORC1 mediates protein synthesis through its two major downstream targets, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K or S6K1). The 4E-BP1 mediates cap-dependent translation, whereas p70S6K mediates ribosome biogenesis (Huang and Houghton 2003).

1.3 mTOR complex 2 (mTORC2) signaling pathway

In addition to mTORC1, mTOR can be in complex with a group of proteins and form mTOR complex 2 (mTORC2). mTORC2 consists of Rapamycin-insensitive companion of mTOR (Rictor) which uniquely exists in mTORC2, mammalian stress-activated map kinase-interacting protein 1 (mSin1) which is essential for Akt phosphorylation, protein observed with Rictor-1 (Protor-1)/Protor-2 and mLST8 (Figure 1.01) (Frias *et al.* 2006; Yang *et al.* 2006; Pearce *et al.* 2007; Zoncu *et al.* 2011).

1.3.1 Upstream regulators of mTORC2 siganling pathway

There has been little information regarding upstream regulator(s) of mTORC2 until recent years. To date, phospholipase D (PLD), TSC1-TSC2 complex and ribosome have been identified as mTORC2 activators, whereas mTORC2 signaling was restrained by glycogen synthase kinase 3β (GSK3β) and p70S6K (Figure 1.02) (Huang *et al.* 2008; Boulbes *et al.* 2010; Julien *et al.* 2010; Chen *et al.* 2011; Zinzalla *et al.* 2011). A recent study of myogenesis mechanism showed that PLD promoted myogenic differentiation through activation of both mTORC1 and mTORC2 in response to vasopressin, albeit mTORC1 acted as negative regulator of myogenesis whereas mTORC2 signaled to PKCα and encouraged myocytic differentiation (Jaafar *et al.* 2011). An *in vitro* study of normal and cancer cells showed that loss of TSC1-TSC2 complex expression resulted in down-regulation of mTORC2 activity. Moreover, the same study showed that the TSC1-TSC2 complex was only co-immunoprecipitated with components of mTORC2 such as

Rictor and mSIN1 whereas Raptor, as the specific feature of mTORC1, was unable to bind to TSC1-TSC2 complex. This suggests that the TSC1-TSC2 complex acts as a positive regulator of mTORC2 (Huang *et al.* 2008). *In vitro* and *in vivo* experiments of yeast and mammalian cells revealed that knockdown of genes encoding 60s or 40s ribosome impaired mTORC2 activity but did not affect mTORC2 assembly. Ribosomes activate mTORC2 activity through binding Rictor and/or mSIN1 in response to insulin-stimulated PI3K signalling (Zinzalla *et al.* 2011). In a recent study, GSK3β was found to inhibit mTORC2/Akt signalling through direct phosphorylation of Ser1235 on Rictor in response to endoplasmic reticulum (ER) stress (Chen *et al.* 2011). Recently, phosphorylation of Thr1135 on Rictor has also been reported by two research groups. However, interpretation of the role of Rictor Thr1135 phosphorylation by these two groups is controversial. Boulbes, D. (2010) reported that the phosphorylation status of Thr1135 on Rictor did not affect mTORC2-dependent phosphorylation of Ser473 on Akt, whereas Julien, L.A. (2010) identified p70S6K as a negative regulator of mTORC2 through phosphorylation of Thr1135 on Rictor, and dephosphorylation of this residue up-regulated mTORC2-mediated phosphorylation of Akt. Although the role of this phosphorylation requires further investigation, both research groups suggest that phosphorylation of Thr1135 on Rictor is linked to the activation status of class I PI3K/mTOR signaling (Boulbes *et al.* 2010; Julien *et al.* 2010).

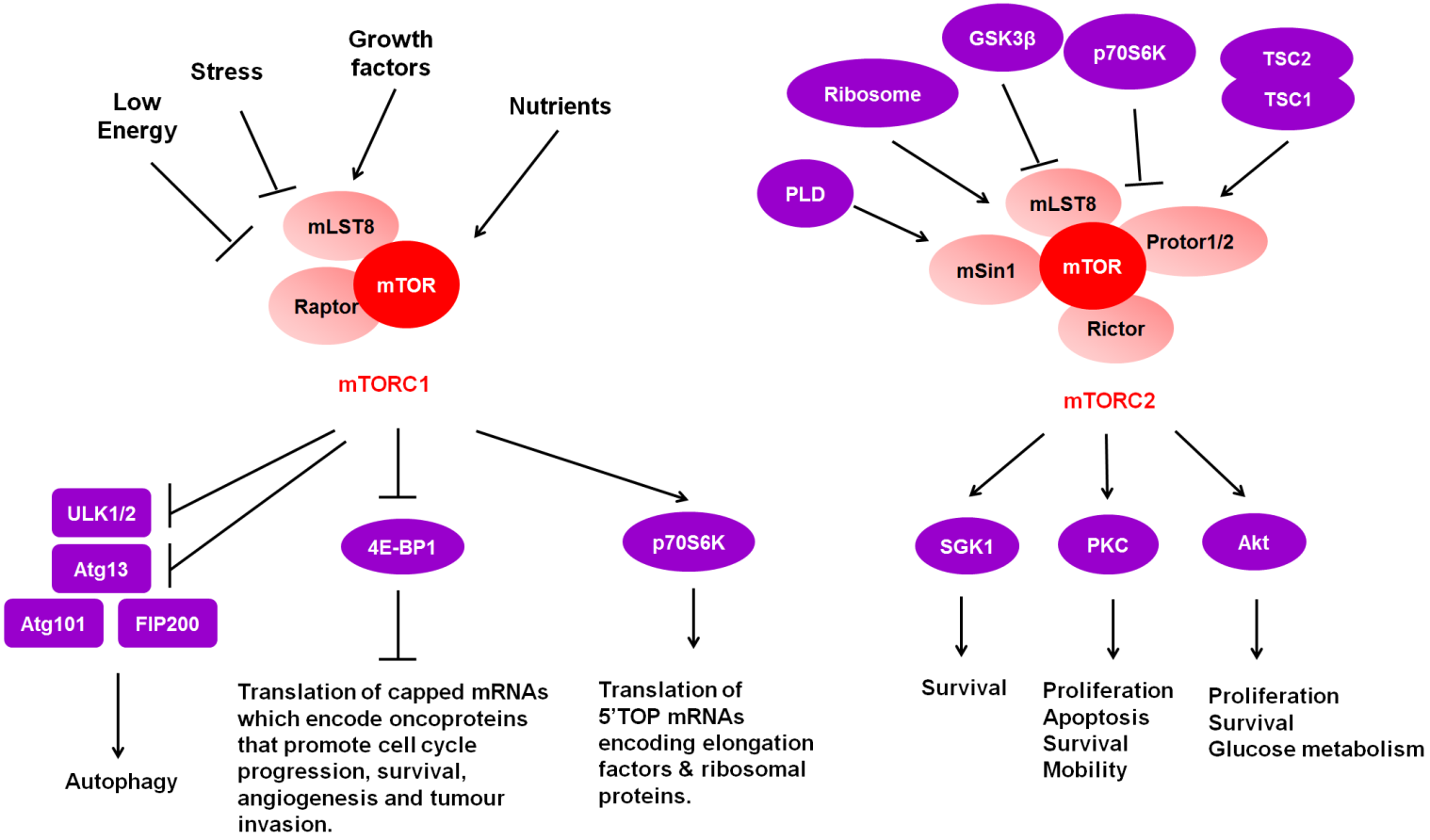


Figure 1.02. An overview of the upstream mTOR signaling factors. Arrows represent active phosphorylation. Bars represent inhibitory phosphorylation. For the full names of the proteins indicated in this figure, see **Lists of Abbreviations**.

1.3.2 Functions and substrates of mTORC2

To date, three kinases, which are Akt, serum and glucocorticoid-inducible kinase (SGK) and protein kinase C (PKC), have recently been identified as downstream targets of mTORC2 (Figure 1.02) (Sarbassov *et al.* 2005; Garcia-Martinez and Alessi 2008; Ikenoue *et al.* 2008). Interestingly, these three proteins are members of the AGC family. mTORC2 positively regulate Akt through phosphorylation of Akt on Ser473. Evidence has shown that simultaneous phosphorylation of both residues can maximally up-regulate Akt activity (Figure 1.02) (Alessi *et al.* 1996). Because Akt has a wide array of substrates, the detail of these substrates and their functions are described in Section 1.3.4. mTORC2 phosphorylates turn motif (TM) and hydrophobic motif (HM) in conventional PKCs (PKCα, PKCβI, PKCβII, PKCγ), and novel PKCε, thus enabling these PKCs to function in a variety of cellular processes such as cell proliferation, apoptosis, survival and mobility (Griner and Kazanietz 2007; Ikenoue *et al.* 2008). mTORC2 activates SGK1 through phosphorylating Ser422 (Garcia-Martinez and Alessi 2008). SGK1 functions in regulation of epithelial ion transport, such as sodium ion, and promotion of cell survival (Loffing *et al.* 2006).

In a recent study, Potor-1 was found to be essential for mTORC2-dependent activation of SGK1, but was not required for regulation of the other two mTORC2 substrates, Akt and PKCα (Pearce *et al.* 2011). Both rictor and mSin1 are important for mTORC2-regulated phosphorylation of Akt, conventional PKCs (PKCα, PKCβ, PKCγ) and novel PKCε, but not other novel PKCs (PKCδ, PKCθ, PKCη) and atypical PKCs (PKCζ, PKCλ). mTOR kinase was found to be involved in mTORC2-dependent phosphorylation of Akt and PKCα (Ikenoue *et al.* 2008).

1.4 Functional domains and organization of class I PI3K, PDK1, Akt, PTEN and mTOR

1.4.1 Class I PI3K

1.4.1.1 Catalytic subunit of class IA PI3K

Class IA PI3K consists of a p110 catalytic subunit and a p85 regulatory subunit. The p110 catalytic subunit has three isoforms p110α, p110β and p110δ, which are encoded by *PIK3CA*, *PIK3CB* and *PIK3CD* genes, respectively. As shown in Figure 1.03, these p110 isoforms of class IA PI3K are characterized by four homology regions (HRs) and a Ras-binding domain, all of which are conserved in the class I PI3K catalytic subunit, and a p85 regulatory subunit-binding domain at N-terminus. The HR1 at the C-terminus is conserved across PI3K, PI4-kinase and PI3K-related kinases such as mTOR, ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) and serves as a domain for Wortmannin (pan-PI3K inhibitor) and catalyzing transfer of the γ phosphate group of ATP to substrate proteins (Wymann and Pirola 1998).

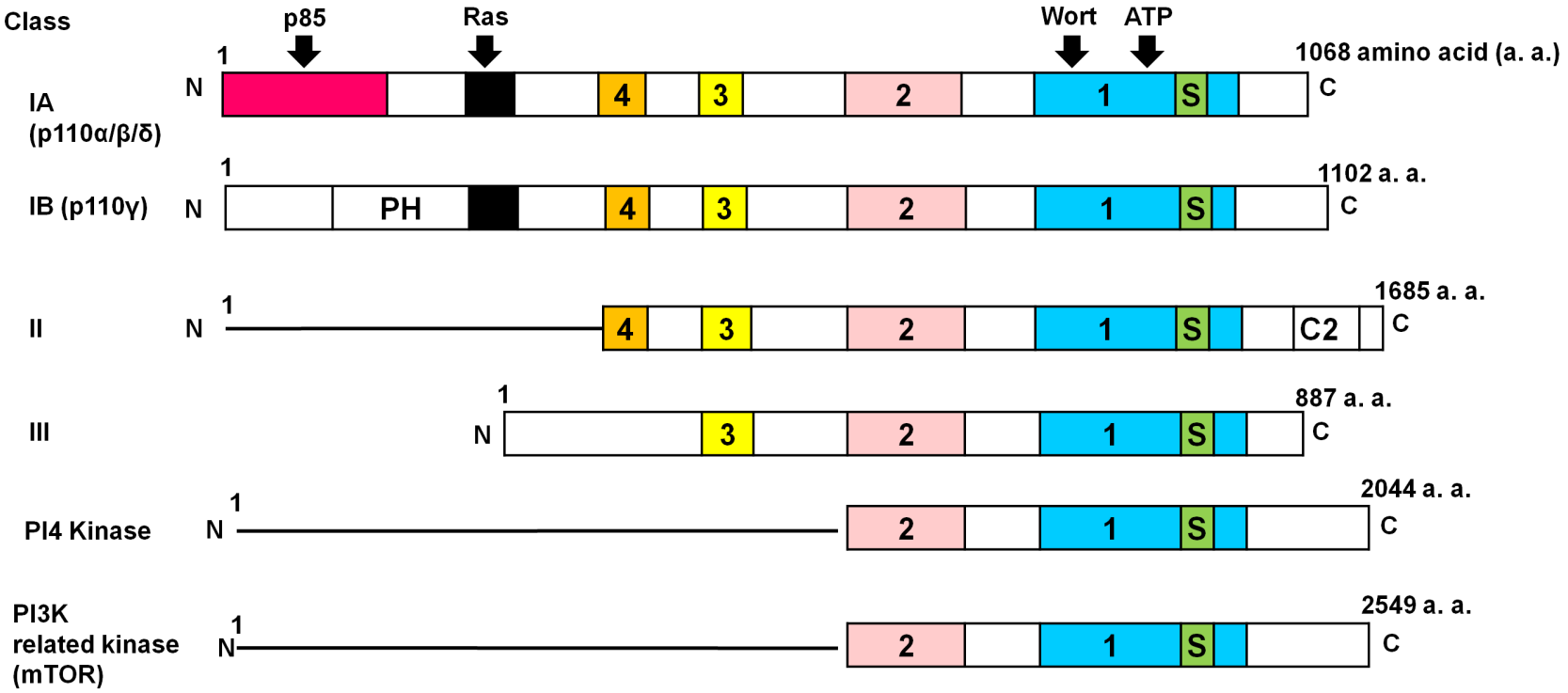


Figure 1.03. Schematic diagram of p110 catalytic subunits of PI3Ks. Number 1-4 represent the conserved homology regions (HR1 to HR4) across all PI3K and related kinases. S represents substrate binding region. The arrows indicate the domains that specifically bind to p85, Ras, Wortmannin (Wort) and ATP respectively. The class IB PI3K contains a putative pleckstry homology (PH) domain. Class II PI3K contains a C2 domain (reproduced from reference (Wymann and Pirola 1998). UniProtKB/Swiss-Prot accession numbers are for p110α catalytic subunit of class IA PI3K: P42336, p110γ catalytic subunit of class IB PI3K: P48736, class II PI3Kα: O00443, class III PI3K: Q8NEB9, PI4- kinase: P42356 and mTOR: P4234.

1.4.1.2 Regulatory subunit of class IA PI3K

The p85 regulatory subunit contains five isoforms p85α, p55α, p50α, p85β and p55γ. The former three isoforms are encoded by *PIK3R1* gene but are expressed in different messenger ribonucleic acid (mRNA) and protein variants through alternative mRNA splicing. The latter two p85 isoforms are encoded by *PIK3R2* and *PIK3R3* genes respectively. All p85 isoforms consist of, starting from N-terminal, a proline-rich region followed by an N-terminal Src homology 2 (NSH2), an inter-SH2 (iSH2) domain and a C-terminal SH2 (CSH2) domain (Figure 1.04). The NSH2 and CSH2 domains can recognize phosphorylated tyrosine (Y) motif, pYXXM, where X represents any amino acid and M is an abbreviation for methionine. The iSH2 domain is the main site for interaction with the three p110 isoforms of class IA PI3K. Both p85α and p85β contain an additional structure located at N-terminus including a Src homology 3 (SH3) domain, a proline-rich region and a breakpoint-cluster-region homology (BH) domain (Wymann and Pirola 1998). The proline rich domains serve as ligands for proteins that contain SH3 domains, such as Abelson (Abl), lymphocyte-specific protein tyrosine kinase (Lck), and p85α and p85β themselves (Kapeller *et al.* 1994). The BH domain has been reported to have GTPase‑activating protein (GAP) activity towards some members of Rho GTPase family, such as Ras-related C3 botulinum toxin substrate 1 (Rac1) and Cell division control protein 42 homolog (Cdc42), which have a role in cytoskeletal rearrangement. However, this binding seems not to be functional (Tolias *et al.* 1995).

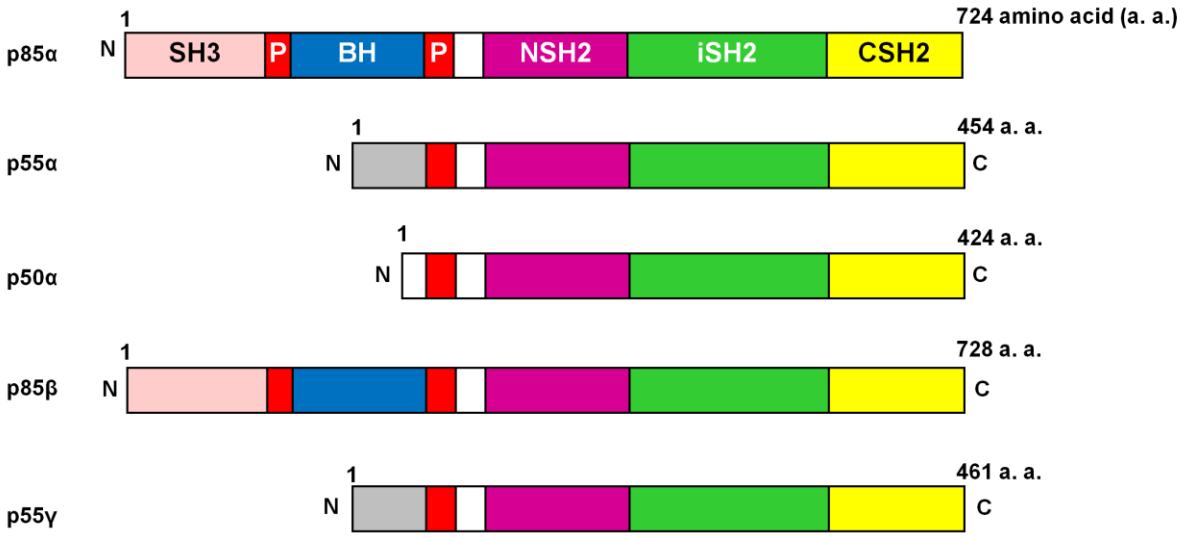


Figure 1.04. Schematic diagram of the isoforms of mammalian class IA PI3K regulatory subunit. p85α, p55α, and p50α are encoded by *PIK3R1* gene, followed by translated from different length of splicing mRNA. p85β and p55γ are encoded by *PIK3R2* and *PIK3R3* genes respectively. SH3 represents Src homology 3 domain. P1 and P2 represent proline-rich domains. BH represents breakpoint-cluster-region (BCR) homology domain. NSH2 represents N-terminal SH2 domain. CSH2 represents C-terminal SH2 domains. iSH2 represents inter-SH2 region (reproduced from reference (Wymann and Pirola 1998)). UniProtKB/Swiss-Prot accession numbers are for p85α, p55α and p50α isoforms, P27986, p85β: O00459 and p55γ: Q92569.

1.4.1.3 Class IB PI3K

Class IB PI3K is a heterodimer that consists of a p110γ catalytic subunit and a p101 or p87/84 regulatory subunit. The p110γ catalytic subunit is encoded by *PIK3CG* gene. As shown in Figure 1.03, the domain structure of p110γ, which is the catalytic subunit of class IB PI3K, is similar to those of the catalytic subunits of class IA PI3K and contains HR1, HR2, HR3 and HR4 domains and Ras-binding domain. Unlike class IA PI3K, p110γ does not have a p85 regulatory subunit-binding domain (Wymann and Pirola 1998; Engelman *et al.* 2006).

Both of the p101 and p87/84 regulatory subunits, which are encoded by *PIK3R5* and *PIK3R6* genes respectively, contain a p110γ-binding domain and a Gβγ-binding domain (Figure 1.05) (Suire *et al.* 2005; Voigt *et al.* 2005; Liu *et al.* 2009). The amino acid sequence of p87/84 is 30% identical to that of p101. The p110γ-p101 heterodimer has a 4-fold higher binding affinity to Gβγ than p110γ-p87/p84 (Suire *et al.* 2005). More recent study suggests that the activation and recruitment of p110γ-p87/p84 heterodimer predominantly relies on the interaction between Ras and p110γ, instead of the weak association of Gβγ with p87/p84 (Kurig *et al.* 2009)

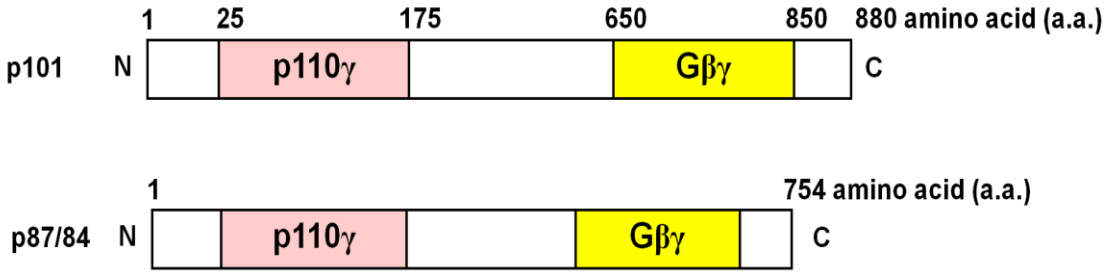


Figure 1.05 Schemetic representing of organization of the isoforms of class IB PI3K regulatory subunits. The p101 and p87/84 regulatory subunits a p110γ-binding domain and a Gβγ-binding domain (Suire *et al.* 2005; Voigt *et al.* 2005; Liu *et al.* 2009). UniProtKB/Swiss-Prot accession numbers are for p101: Q8WYR1, and for p87/84: Q5UE93.

1.4.2 PDK1

PDK1, which is encoded by *PDPK1* gene, contains a catalytic domain that catalyzes transfer of the γ phosphate group from ATP to a protein substrate, and a PH domain which allows PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 to recognize and recruit to inner side of plasma membrane (Figure 1.06) (Alessi *et al.* 1997). It has been reported to be constitutively activated in cytosol due to its auto-phosphorylation on Ser241 and its activity can be up-regulated up to ~1000 fold following PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3-mediated cytoplasmic membrane localization (Casamayor *et al.* 1999).

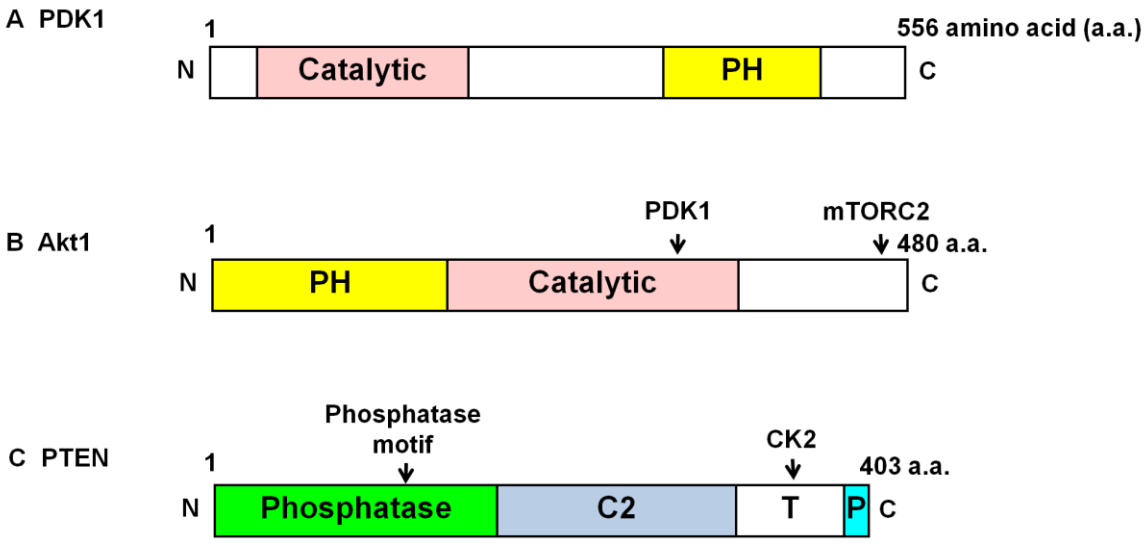


Figure 1.06. Schematic diagram of PDK1, PTEN and Akt1 kinases. Both of PDK1 (A) and Akt1 (B) contain a Catalytic domain that catalyzes transfer of a phosphate group from ATP to a protein substrate and a pleckstry homology (PH) domain which allows PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 to recognize and recruit to inner side of plasma membrane. (C) PTEN contains an N-terminal Phosphatase domain with a phosphatase motif which can catalyze and release a 3' phosphate group from PtdIns(3,4,5)P_3, a C2 domain that allows PTEN association with plasma membrane, a tail (T) region that contains CK2 phosphorylation sites and a C-terminal PDZ domain that facilitates PTEN association with MAGI proteins (Alessi *et al.* 1997; Simpson and Parsons 2001; Du and Tsichlis 2005). UniProtKB/Swiss-Prot accession numbers are for PDK1: O15530, Akt1: P60484 and PTEN: P31749.

1.4.3 Akt kinase family

The Akt kinases have three isoforms Akt1, Akt2 and Akt3 that are encoded by *Akt1*, *Akt2* and *Akt3* genes, respectively. These Akt isoforms shares similar domain structure consisting of an N-terminal PH domain for PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 binding, a catalytic domain that can phosphorylate a given substrate protein through catalyzing transfer of the γ phosphate group from ATP to substrate proteins, and a C-terminal extension (Figure 1.06) (Kumar and Madison 2005).

To date, two major phosphorylation sites have been identified for Akt activation. Whilst PDK1 typically phosphorylates Thr308 on Akt1, Thr309 on Akt2, and Thr305 on Akt3, mTORC2 phosphorylates Ser473 on Akt1, Ser474 on Akt2 and Ser472 on Akt3 (Alessi *et al.* 1996; Brodbeck *et al.* 1999; Baer *et al.* 2005; Sarbassov *et al.* 2005). Evidence has shown that simultaneous phosphorylation of both residues can maximally up-regulate Akt activity (Alessi *et al.* 1996).

1.4.4 PTEN

The PTEN suppressor is encoded by the *PTEN* gene and is composed of an N-terminal phosphatase domain for dephosphorylation of PtdIns(3,4,5)P_3, a C2 domain that allows PTEN to anchor phospholipid membrane, a C-tail region which contains two PEST sequences for proteolysis of PTEN substrates and several CK2 phosphorylation sites (Ser380, Thr382, Thr383, and Ser385) for promoting PTEN stabilization, and a C-terminal PDZ motif that interacts with other proteins such as membrane associated guanylate kinase inverted (MAGI) proteins (Figure 1.06) (Simpson and Parsons 2001).

1.4.5 mTOR

mTOR belongs to a phosphinositide-3-kinase-related kinase (PIKK) family containing catalytic kinase domains for phosphorylation of substrate proteins and large molecular weights of ~300-500 kDa. mTOR consists of, starting from N-terminus, two

HEAT (for huntingtin elongation factor 3, a subunit of protein phosphatase 2A and TOR1) repeat domains that are associated with the interaction between mTOR and other proteins, a FAT (for FRAP, ATM and TRRAP) domain which is conserved in the phosphinositide-3-kinase-related kinase (PIKK) family, a FKBP12-rapamycin binding (FRB) domain which is a unique feature of mTOR explaining the high specificity for Rapamycin (an mTOR inhibitor). Immediate downstream of the FRB domain resides the PIKK-related kinase domain essential for phosphorylation of downstream targets, then the FATC (FAT-C) domain which is homologous to the large N terminal portion of the FAT region. Co-existence of FAT and FATC domains implies that their interaction contributes to the complete function of the kinase domain (Figure 1.07) (Bosotti *et al.* 2000; Chiang and Abraham 2007; Tsang *et al.* 2007).

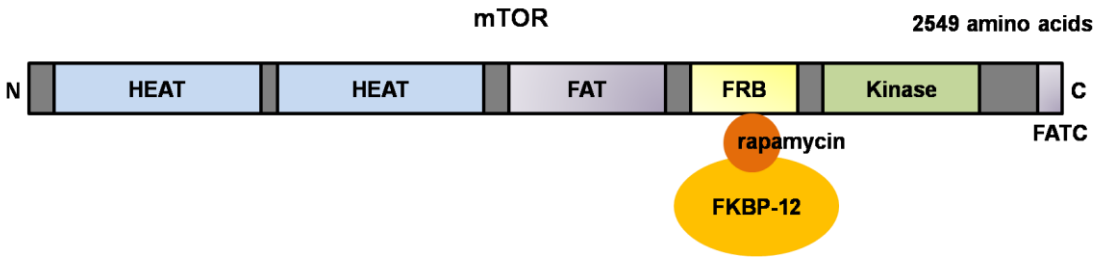


Figure 1.07. Schematic diagram of mTOR structural organization. The mTOR consists of, staring from N-terminus, two HEAT (for huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1) repeat domains, followed by a FAT (for FRAP, ATM and TRRAP) domain, a FRB (for FKBP12-rapamycin binding) domain, a kinase domain, and a C-terminal FATC (for FAT-C). Rapamycin, a product of the bacterium *Streptomyces hygroscopicus*, is the first indentified compound that specifically targets TOR in yeast and mTOR in mammals. The mechanism of Rapamycin inhibition of mTOR kinase activity is initially to bind to cytosolic receptor FKBP-12. Subsequently, the Rapamycin-FKBP-12 complex targets the FRB domain in mTOR (Huang *et al.* 2003; Chiang and Abraham 2007).

1.5 Class I PI3K/Akt/mTOR signaling network

1.5.1 Substrates and functions of class I PI3K

Class I PI3K can exert cellular functions after it converts PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Although both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are able to recognize and interact with proteins containing PH domain, PtdIns(3,4,5)P_3 only recognizes PH domain whereas PtdIns(4,5)P_2 has multiple targets in addition to PH domain-containing proteins. Currently, there are approximately 250 PH domain-containing proteins in the

human genome database but most of their functions remain unresolved (Lemmon 2003). Table 1.1 summarizes the functions of the identified proteins which contain PH domain. Most cellular effects exerted by class I PI3K is through PDK1/Akt pathway and the functions of PDK1 and Akt will be described in Section 1.5.2 and 1.5.3. Besides, class I PI3K promotes cell migration through PtdIns(3,4,5)P_3-recruited PH-containing proteins such as Rac and adenosine diphosphate (ADP)-ribosylating factor 6 (Arf6) (Cantley 2002; Kamura *et al.* 2010).

Table 1.1 Current identified pleckstrin homology (PH) domain-containing downstream effectors of PtdIns(3,4,5)P_3

Protein with pleckstrin homology (PH) domain	Function	Reference
PDK1	PDK1 activates Akt and other member proteins of AGC serine/threonine kinase family.	(Pearce *et al.* 2010)
Akt	Akt promotes cellular proliferation, survival and glucose metabolism.	(Whiteman *et al.* 2002; Altomare and Testa 2005)
Rac1	Rac1 stimulates angiogenesis through increasing expression levels of Hypoxia-inducible factors- 1α (HIF-1α) and VEGF, and promotes cytoskeletal rearrangement for cell mobility	(Cantley 2002; Xue *et al.* 2011)
Arf6	Arf6 promotes cytoskeletal rearrangement for cell mobility	(Cantley 2002)

Table 1.1 Current identified pleckstrin homology (PH) domain-containing downstream effectors of PtdIns(3,4,5)P_3 *continued*

Protein with pleckstrin homology (PH) domain	Function	Reference
Cytohesin family	Cytohesin family is a GDP/GTP exchange factor for Arf and functions in promoting Arf-mediated vesicular trafficking and cytoskeletal rearrangement, and inhibiting thyroid hormone signaling	(Jackson *et al.* 2000; Poirier *et al.* 2005)
Tyrosine-protein kinase (Tec) family members: 1. Tec 2. Bruton's tyrosine kinase (Btk) 3. IL2-inducible T-cell kinase (Itk) 4. Bone marrow tyrosine kinase gene in chromosome X protein (Bmx)	Tec family proteins function in development of lymphocytes and other hematopoietic cells, such as phospholipase C-γ (PLC-γ)-activated calcium mobilization, activation of mitogen-activated protein kinase (MAPK) signaling, cytoskeleton rearrangements, Fas-mediated apoptosis, etc.	(Takesono *et al.* 2002)

Table 1.1 Current identified pleckstrin homology (PH) domain-containing downstream effectors of PtdIns(3,4,5)P_3 *continued*

Protein with pleckstrin homology (PH) domain	Function	Reference
Dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1)	The functions of DAPP1 are involved in the development of B lymphocytes through regulation of cell growth and survival, cytoskeletal rearrangement, PLC-2 γ activation and calcium mobilization.	(Zhang *et al.* 2009)
Grb 2-associated binder 1/2/3 (Gab1/2/3)	Gab proteins promote activation of PI3K signaling pathway. Gab1 functions in termination of PI3K activity but activation of Ras/ extracellular signal-regulated kinase (Erk) signaling when Gab1 interacts with SH2 domain-containing tyrosine phosphatase 2 (Shp-2). By contrast, Gab2 simultaneously activates both PI3K and Erk pathways.	(Mattoon *et al.* 2004; Sampaio *et al.* 2008; Zhang *et al.* 2009)

Table 1.1 Current identified pleckstrin homology (PH) domain-containing downstream effectors of PtdIns(3,4,5)P_3 *continued*

Protein with pleckstrin homology (PH) domain	Function	Reference
Centaurin	Centaurin inactivates Arf-mediated vesicular trafficking and cytoskeletal rearrangement.	(Jackson *et al.* 2000)
Src kinase-associated phosphoprotein (SKAP)	SKAP activates integrin lymphocyte function-associated antigen 1 (Lfa-1) and promotes cytoskeletal rearrangement of T lymphocytes	(Swanson *et al.* 2008)
Adhesion and degranulation-promoting adapter protein (ADAP)	ADAP activates integrin Lfa-1 and promotes cytoskeletal rearrangement of lympho-hematopoietic cells	(Swanson *et al.* 2008)

Table 1.1 Current identified pleckstrin homology (PH) domain-containing downstream effectors of PtdIns(3,4,5)P_3 *continued*

Protein with pleckstrin homology (PH) domain	Function	Reference
Vav family protein (Vav1/2/3)	Vav family protein is a GDP/GTP exchange factor for Rac. Vav can stimulate Rac-mediated cytoskeletal rearrangement and cell motility. Vav plays a role in development of T lymphocytes through activation of T cell antigen receptor (TCR) downstream signaling, including calcium mobilization, activation of Erk and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, integrin Lfa-1 activity, cellular polarization and cytoskeletal rearrangement .	(Han *et al.* 1998; Tybulewicz 2005)

1.5.2 Substrates and functions of PDK1

It has been known that the major substrate of PDK1 is Akt. Both PDK1 and Akt are members of the AGC serine/threonine kinase family characterized by possession of a catalytic kinase domain resembling cAMP-dependent protein kinase 1 (PKA), PKC and cGMP-dependent protein kinase (PKG) (Pearce *et al.* 2010). The functions of Akt will be described in Section 1.5.3. In addition to Akt, PDK1 has been reported to phosphorylate and activate more than 23 protein kinases belonging to AGC kinase family, including p70S6K, 90 kDa ribosomal S6 kinase (RSK), PKC, PKC-related protein kinase (PKN) and SGK (Pearce *et al.* 2010).

There are two approaches for PDK1 to interact with and activate its downstream targets. For Akt that contain a PH domain, alteration of the conformation of Akt occurs after it is recruited by PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 to the inner side of cell membrane. This allows PDK1 to phosphorylate and activate Akt (Calleja *et al.* 2007). The mechanism for PDK1 to phosphorylate and activate the substrates without a PH domain, such as p70S6K and SGK, relies on the interaction between the PIF-binding pocket in the catalytic domain of PDK1 and the hydrophobic T-loop motif of p70S6K and SGK (Biondi *et al.* 2001). Table 1.2 summarizes the functions of these PDK1 downstream targets.

Table 1.2 Downstream effectors of PDK1

PDK1 substrate	Substrate and Function	Reference
Akt	See Chapter 1, Section 1.5.3 Akt kinase	
p70S6K or S6K1	(1) S6RP, eIF4B, eEF2K, Pdcd4: promote protein synthesis (2) Rac, CDC42: cytoskeletal rearrangement (3) IRS-1: negative feedback loop of insulin/insulin-like growth factor signaling (4) mTOR: positive feedback regulation (5) 46 kDa DNA polymerase delta interaction protein (SKAR): cell growth (6) Rictor: negative feedback loop of mTORC2 signaling	(Fenton and Gout 2011)

Table 1.2 Downstream effectors of PDK1 *continued*

PDK1 substrate	Substrate and Function	Reference
RSK	(1) Eukaryotic translation initiation factor 4B (eIF4B), eukaryotic elongation factor 2 kinase (eEF2K), S6 ribosomal protein (S6RP), TSC2: promote protein synthesis	(Anjum and Blenis 2008)
	(2) Nuclear factor of activated T cells 3 (NFAT3), cAMP response element-binding (CREB): promote transcription	
	(3) Cyclin-dependent kinase inhibitor 1B ($p27^{KIP}$), GSK-3β, MAX dimerization protein 1 (Mad1): promote cell cycle progression	
	(4) B-cell lymphoma-2 (Bcl-2) antagonist of cell death (Bad), CCAAT/enhancer binding protein-β (C/EBPβ), CREB, inhibitor of the nuclear factor of kappa light polypeptide gene enhancer in B-cells-alpha (IκBα): maintain cell survival	
	(5) Filamin A (FLNA): cytoskeletal rearrangement	

Table 1.2 Downstream effectors of PDK1 *continued*

PDK1 substrate	Substrate and Function	Reference
PKN	Tau: α-actinin cross-linking actin and intermediate filament proteins: promote the assembly and stabilization of cytoskeletal proteins	(Kawamata *et al.* 1998)
PKC	(1) Adducin 1: cytoskeletal rearrangement (promote cell mobility) (2) Glycoprotein 130 (gp130), C/EBPβ, C-reactive protein (CRP), epidermal growth factor receptor (EGFR), cluster of differentiation 64 (CD64), Jun B: tumour progression and metastasis (3) G protein–coupled receptor kinase 2 (GRK2): cardiac hypertrophy due to down-regulation of β-adrenergic receptor signaling (4) GSK3: promote cell cycle progression (5) Protein kinase D (PKD): promote Erk signaling, cell survival and cell vesicle trafficking (6) Phosphodiesterase 3a (PDE3a): increase cAMP level	(Chen *et al.* 2007; Hunter *et al.* 2009; Malhotra *et al.* 2010; Hafeez *et al.* 2011; Mole *et al.* 2011; Rozengurt 2011)

1.5.3 Functions and substrates of Akt

Once Akt is activated, it can phosphorylate a wide array of substrates which engage activities of normal cell development and tumourigenicity. Previous studies of the roles of Akt isoforms in normal cell development suggest that Akt1 preferentially functions in promoting growth, proliferation and survival of cells, whereas Akt2 is involved in utilizing blood glucose in response to insulin and maintenance of glucose homeostasis. Akt3 is involved in stimulation of cell growth (Chen *et al.* 2001; Cho *et al.* 2001; Cho *et al.* 2001; Easton *et al.* 2005).

1.5.3.1 Akt promotes G1/S cell cycle progression

To date, Akt has been shown to stimulate G1/S cell cycle progression through inhibitory phosphorylation of cell cycle inhibitors $p21^{WAF1}$and $p27^{KIP}$ (Figure 1.08) (Zhou *et al.* 2001; Liang *et al.* 2002). Akt phosphorylation of glycogen synthase kinase 3α (GSK-3α) and GSK-3β prevents ubiquitin-modulated proteolysis of cyclin D1, thus maintaining activity of cyclin D1 and promoting G1/S phase transition (Figure 1.08) (Cross *et al.* 1995). Akt has been reported to induce cell cycle progression to S phase through phosphorylation and inhibition of several Forkhead box (Fox) transcription factors (Figure 1.08). For instance, Akt can inhibit Forkhead box O4 (FoxO4)-induced transcription and translation of $p27^{KIP}$ (Medema *et al.* 2000). Akt also counteracts functions of Forkhead box O1a (FoxO1a), Forkhead box O3a (FoxO3a) and FoxO4, thereby down-regulating levels of cyclin D1 and cyclin D2 proteins (Figure 1.08) (Schmidt *et al.* 2002; Ho *et al.* 2008).

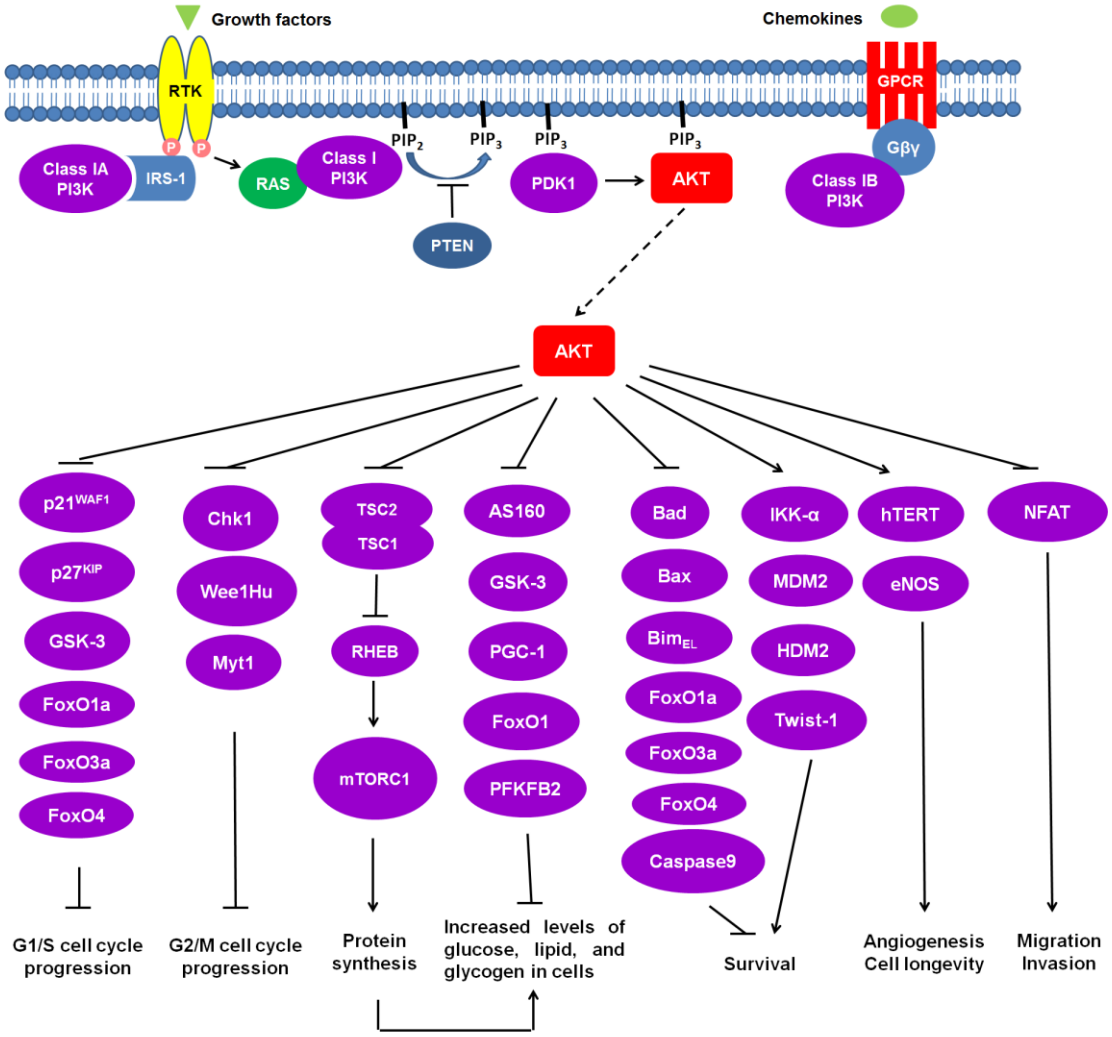


Figure 1.08. Akt signaling pathways. Simultaneous PDK1- and mTORC2-mediated phosphorylation of Akt kinases drives full activition of Akt kinases, allowing Akt to phosphorylate its wide array of substrates, resulting in promotion of cell proliferation, survival, glucose uptake and prevention of glucose from re-entering blood stream, and exacerbation of malignant phenotype in tumour cells (Whiteman *et al.* 2002; Altomare and Testa 2005). Arrows represent active phosphorylation. Bars represent inhibitory phosphorylation. PIP2, phosphatidylinositol-4,5–biphosphate; PIP3, phosphatidylinositol-3,4,5–triphosphate. For the full names of the proteins indicated in this figure, see **Lists of Abbreviations**.

1.5.3.2 Akt promotes G2/M cell cycle progression

Akt has been reported to promote G2/M cell cycle progression through direct phosphorylation of substrates including checkpoint kinase 1 (Chk1), Wee1 homolog in *S. pombe* (Wee1Hu), myelin transcription factor 1 (Myt1), and FoxO transcription factors (Figure 1.08) (Okumura *et al.* 2002; Schmidt *et al.* 2002; Katayama *et al.* 2005; Puc *et al.* 2005; Ho *et al.* 2008). Akt-mediated phosphorylation of Chk1, a DNA damage checkpoint kinase, promotes nuclear exportation and ubiquitin-modulated proteolysis of ChK1 and facilitates G2/M phase transition (Figure 1.08) (Puc *et al.* 2005). Akt activates cyclin-dependent kinase (Cdc2)-mediated G2/S phase progression through inhibitory phosphorylation of Wee1Hu, which is an inhibitor of Cdc2 (Figure 1.08). Akt-mediated phosphorylation of Wee1Hu on Ser642 facilitates Wee1Hu binding to 14-3-3θ protein, which results in nuclear export of Wee1Hu by 14-3-3θ (Katayama *et al.* 2005). In a starfish model, Akt directly phosphorylates and inactivates Myt1, leading to an increase in phosphorylation and activation of Cdc2/cyclinB (Figure 1.08) (Okumura *et al.* 2002).

1.5.3.3 Anti-apoptotic activity of Akt

A large body of literature has demonstrated Akt to be a critical survival factor. Akt has been shown to antagonize apoptotic activity through direct phosphorylation of several substrates, including FoxO1a, FoxO3a, FoxO4, Bad, caspase 9, BCL2-associated X protein (Bax) and the EL isoform of Bcl-2-like protein 11 (Bim_{EL}) (Figure 1.08) (Datta *et al.* 1997; Cardone *et al.* 1998; Brunet *et al.* 1999; Tsuruta *et al.* 2002; Gardai *et al.* 2004; Qi *et al.* 2006; van der Vos and Coffer 2011). In addition to the arrest of G1/S phase transition as mentioned above, FoxO1a, FoxO3a and FoxO4 can induce apoptosis through stimulation of pro-apoptotic transcriptional targets, including Bcl-2-like protein 11 (Bim), p53 upregulated modulator of apoptosis (PUMA), B-cell lymphoma-6 (Bcl-6), PTEN-induced kinase 1 (Pink1), Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) and tumor necrosis factor receptor-associated death domain (TRADD) (Brunet *et al.* 1999; Modur *et al.* 2002; Rokudai *et al.* 2002; Tang *et al.* 2002; Gilley *et al.* 2003; You *et al.* 2006; Mei *et al.* 2009). The Akt-mediated

phosphorylation of these FoxO factors promotes nuclear exportation and cytoplasmic retention of these substrates, which prevents them from binding to their target genes in the nucleus and initiating transcription of pro-apoptosis genes as mentioned above (Figure 1.08) (van der Vos and Coffer 2011). Activation of extrinsic apoptotic signals such as Fas and TNF induces Bax translocation from the cytoplasm to mitochondria, causing the mitochondrial outer membrane to become permeable, allowing the release of cytochrome c into the cytosol and subsequent activation of caspase 9, caspase 3 and caspase 7. Activation of these caspases directs the cell to apoptosis (Jiang and Wang 2004). Akt-mediated phosphorylation of Bax prevents movement of Bax into mitochondria and inhibits apoptosis (Figure 1.08) (Tsuruta *et al.* 2002; Gardai *et al.* 2004). Bad is usually dephosphorylated in response to apoptotic stimuli. The dephosphorylated Bad can then be released from 14-3-3, allowing free Bad to translocate to the mitochondria where it can form a heterodimer with two anti-apoptotic factors, Bcl-2 and B-cell lymphoma-extra large (Bcl-xL), blocking their functions and thereby facilitating Bax/Bcl2-antagonist/killer 1(Bak)-induced apoptosis (Adachi and Imai 2002). Akt phosphorylates Bad, thus countering Bad-triggered pro-apoptotic activity (Figure 1.08) (Datta *et al.* 1997; Brunet *et al.* 1999). Moreover, earlier studies found that the activity of pro-apoptotic factor Bim was predominantly regulated by the v-raf murine sarcoma/leukemia viral oncogene homolog (Raf)/Erk pathway, which functioned in inducing ubiquitination-proteolysis of Bim. In normal cells, Bim is bound to microtubule-associated dynein light chain 8 kDa (LC8) to form Bim-LC8 complex. After receiving apoptotic stimuli, Bim starts to dissociate from Bim-LC8 complex. The free Bim can interact with two anti-apoptotic factors Bcl-2 and B-cell lymphoma-related gene (Bcl-X) and counteracts pro-apoptotic activity. However, a more recent study shows that phosphorylation of Ser87 in Bim_{EL} by Akt facilitates 14-3-3 interaction with Bim_{EL}, resulting in ubiquitination-proteolysis of Bim_{EL} and promotion of cell survival (Figure 1.08) (Qi *et al.* 2006).

1.5.3.4 Pro-survival activity of Akt

In addition to antagonizing pro-apoptotic activities, Akt can phosphorylate pro-survival factors for maintaining cell survival, such as inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK-α), murine double minute 2 (MDM2), MDM2 in human (HDM2) and twist homolog 1 in *Drosophila* (Twist-1) (Figure 1.08) (Ozes *et al.* 1999; Ma *et al.* 2009; Vichalkovski *et al.* 2010; Fenouille *et al.* 2011). Activation of PI3K/Akt signaling allows Akt to phosphorylate IKK-α, which in turn up-regulates the pro-survival transcription factor NF-κB, thus promoting transcription of anti-apoptotic genes (Ozes *et al.* 1999). Activated Akt phosphorylates MDM2 in murine and HDM2 in human, which in turn promotes ubiquitination-mediated proteolysis of p53 tumour suppressor and $p21^{WAF1}$, resulting in down-regulation of p53-mediated transcription of pro-apoptotic factors such as Bax and PUMA, and of cell cycle arrest protein $p21^{WAF1}$ in response to DNA damage (Alarcon-Vargas and Ronai 2002; Ma *et al.* 2009; Fenouille *et al.* 2011). A recent study also suggests that direct phosphorylation of Twist-1 by Akt impairs transcriptional activity of p53 in response to DNA damage and stress stimuli (Vichalkovski *et al.* 2010).

1.5.3.5 Akt-regulated glucose metabolism

It is evident that insulin regulates blood glucose homeostasis through activation of insulin receptor (IR) and subsequent stimulation of class I PI3K/Akt signaling. Under normal conditions, elevated blood glucose stimulates secretion of insulin, which can promote adipocytes and skeletal myocytes to take up glucose from the blood stream, stimulate glycogen synthesis and inhibit glycogenolysis in the liver and skeletal muscle. Insulin also reduces glucose generation (gluconeogenesis) in the liver and blocks glucose release, as well as accelerating the conversion of glucose to fatty acid (lipogenesis) and suppressing lipolysis (Whiteman *et al.* 2002). It has been observed that the insulin-activated Akt promotes blood glucose uptake into cells through inhibitory phosphorylation of a 160 kDa Akt substrate (AS160), a negative regulator of glucose transporter type 4 (GLUT4) that functions in carrying glucose into cells (Figure 1.08)

(Eguez *et al.* 2005). Akt can increase glucose uptake into cells by up-regulation of glucose transporter type 1 (GLUT1) at the transcription and translation level through Akt/mTORC1-activated cap-dependent translation, resulting in an increase in expression levels of GLUT1 and hypoxia-inducible factor (HIF). The increased HIF subsequently promotes transcription of the gene encoding GLUT1 (Zelzer *et al.* 1998; Taha *et al.* 1999). GSK-3 has been found to down-regulate glycogen synthesis through inhibitory phosphorylation of glycogen synthase. GSK-3 is also involved in reducing lipogenesis through GSK-3-dependent phosphorylation of sterol regulatory element binding protein (SREBP) transcription factor, which in turn promotes ubiquitination-proteolysis of SREBP, resulting in down-regulation of SREBP-modulated transcription of target genes that promote lipogenesis. Alternatively, GSK-3 phosphorylates and inhibits ATP citrate lyase (ACL)-mediated generation of acetyl-CoA, a source of acetyl-CoA for lipogenesis. Hence, Akt-mediated phosphorylation and inhibition of GSK-3 allows activation of glycogen synthesis and lipogenesis (Figure 1.08) (Potapova *et al.* 2000; Sundqvist *et al.* 2005; Patel *et al.* 2008). Akt can inhibit gluconeogenesis and lipolysis in the liver through phosphorylation and inhibition of peroxisome proliferative activated receptor γ coactivator (PGC-1) ((Figure 1.08) (Li *et al.* 2007). As FoxO1 can up-regulate transcription of glucose-6-phosphatase (G6Pase), which acts as an activator of gluconeogenesis, Akt can down-regulate gluconeogenesis through inhibition of FoxO1 activity (Figure 1.08) (Nakae *et al.* 2001). The third way for Akt to inhibit hepatic gluconeogenesis is through direct phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2) which positively regulates glucose uptake into cells and lipogensis (Figure 1.08) (Moon *et al.* 2011).

1.5.3.6 Cross-talk of Akt with components of Raf/Erk pathway

In addition to the regulation of multiple cellular processes, Akt has been demonstrated to cross-talk with Raf/Erk signaling through inhibitory phosphorylation of Raf, which in turn may generate cell-specific outcomes (Zimmermann and Moelling 1999; Guan *et al.* 2000; Reusch *et al.* 2001). For example, the Akt-mediated inhibition

of Raf results in down-regulation of the Raf/mitogen-activated protein kinase (Mek)/Erk pathway and promotes cell cycle progression in HEK293 cells (Zimmermann and Moelling 1999). Another study of vascular smooth muscle (VSM) cells showed that platelet-derived growth factor (PDGF) is a potent mitogen which can activate Akt, which in turn strongly phosphorylates and inhibits Raf activity and blocks the downstream Mek/Erk signaling, resulting in proliferation of VSM cells. Conversely, Akt only weakly interacted with Raf in response to thrombi, which subsequently activated Raf/Mek/Erk signaling, leading to re-differentiation of VSM cells (Reusch *et al.* 2001).

1.5.3.7 Dysregulation of Akt signaling drives tumorigenesis

Accumulating evidence suggests that Akt regulates cellular processes that promote tumourigenicity. For example, Akt promotes vasodilation and angiogenesis through phosphorylation of nitric oxide synthase 3 in endothelial cells (eNOS) which subsequently activates eNOS-mediated production of nitric oxide (NO) in vascular endothelium. The elevated NO can stimulate dilation of blood vessel and angiogenesis (Figure 1.08) (Dimmeler *et al.* 1999). Akt also induces angiogenesis through Akt/mTORC1-mediated cap-dependent translation that synthesizes HIF transcription factor. The production of HIF up-regulates transcription of target genes associated with angiogenesis, such as vesicular endothelial growth factor (VEGF) and endoglin (ENG) (Semenza 2003). Akt has been found to promote longevity of cancer cells through phosphorylating and activating human telomerase reverse transcriptase (hTERT), which prolongs cellular lifespan through maintaining DNA telomere length (Figure 1.08) (Liu 1999). Akt1 has been described acting as an inhibitor of cellular motility and invasion through Akt-dependent phosphorylation of HDM2 and subsequent down-regulation of HDM2-modulated ubiquitination-proteolysis of nuclear factor of activated T cells (NFAT), which functions in induction of motility and invasion of cancer cells (Figure 1.08) (Yoeli-Lerner *et al.* 2005). Previous findings showed that Akt1 also inhibited cell motility and migration through down-regulation of Erk-modulated epithelial-mesenchymal transition (EMT). By contrast, Akt2 was reported to play a role opposite

to Akt1 and acted as a positive regulator of EMT in response to growth factor stimuli (Irie *et al.* 2005; Lee *et al.* 2011).

1.5.4 Upstream inputs of mTORC1

1.5.4.1 Growth factors and cytokines regulate mTORC1

It has been well established that the predominant upstream regulator of mTORC1 is the class I PI3K/Akt signaling pathway. Members of the Raf/Erk MAPK pathway have also been reported to positively regulate mTORC1 signaling through targeting TSC2. ERK and its substrate p90RSK have been found to phosphorylate Ser664 and Ser1798 on TSC2, respectively. This in turn inhibits the GAP activity of TSC complex towards the Rheb/mTORC1 pathway, resulting in activation of mTORC1 (Figure 1.09) (Roux *et al.* 2004; Ma *et al.* 2007). Another study showed that p38 MAPK activated mTORC1 through its substrate MAPK-activated protein kinase-2 (MK2), which phosphorylated Thr1210 on TSC2, which in turn facilitated 14-3-3 binding to TSC2 and thereby inhibited TSC2 activity (Figure 1.09) (Li *et al.* 2003). Recently, IKK-α has been demonstrated to act as a transducer of cellular response to tumour necrosis factor-alpha (TNF-α) and activate mTORC1 through inhibitory phosphorylation of Ser487 and Ser511 on TSC1 (Figure 1.09) (Lee *et al.* 2007).

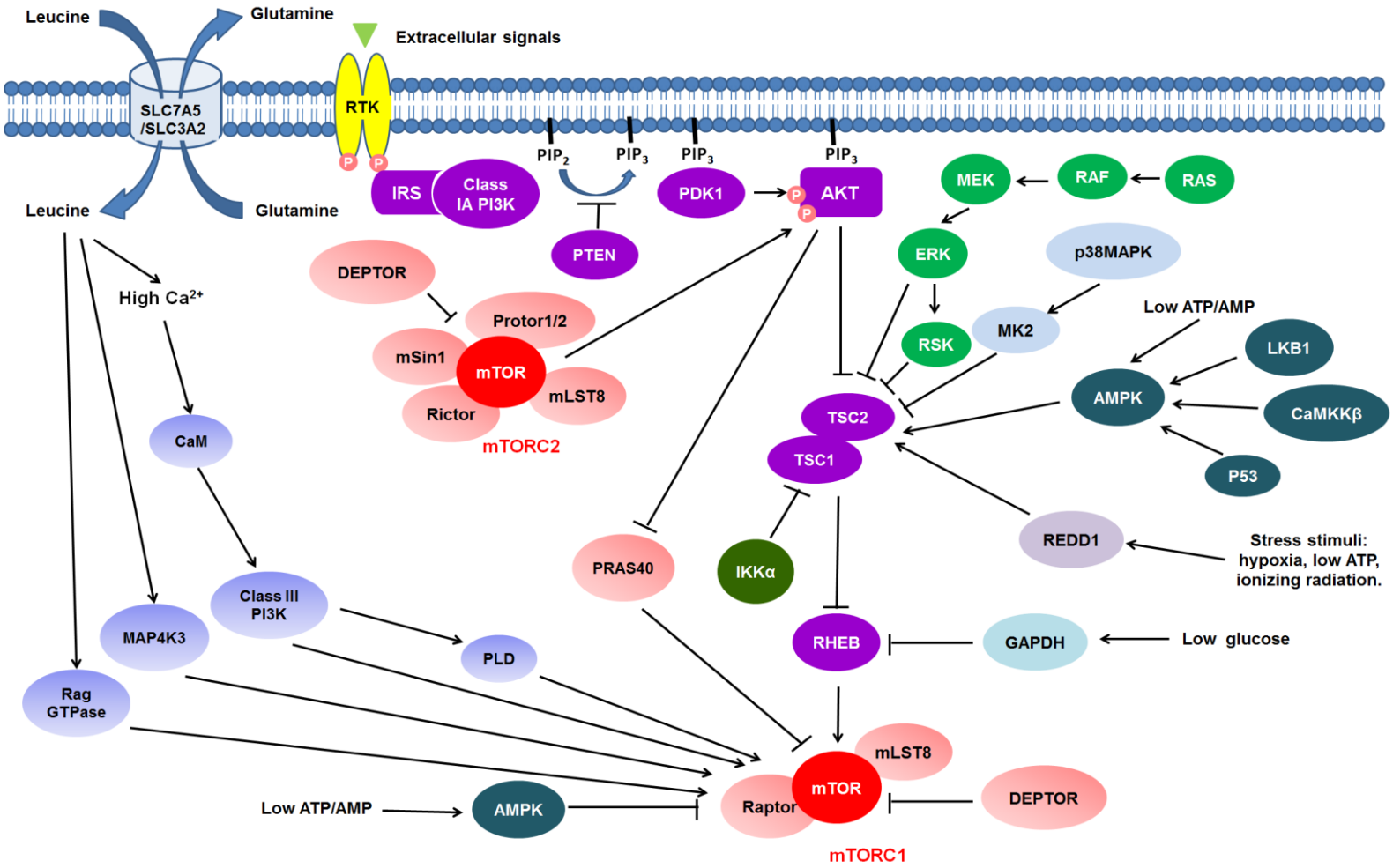

Leucine
Glutamine
Extracellular signals
SLC7A5
/SLC3A2
RTK
Leucine
Glutamine
IRS
Class
IA PI3K
PIP2
PIP3
PIP3
PIP3
PDK1
AKT
PTEN
MEK
RAF
RAS
ERK
p38MAPK
DEPTOR
Protor1/2
mSin1
mTOR
mLST8
Rictor
mTORC2
High Ca2+
CaM
RSK
MK2
Low ATP/AMP
LKB1
AMPK
CaMKKβ
P53
TSC2
TSC1
REDD1
Stress stimuli:
hypoxia, low ATP,
ionizing radiation.
PRAS40
IKKα
Class III
PI3K
MAP4K3
PLD
RHEB
GAPDH
Low glucose
Rag
GTPase
mLST8
mTOR
Raptor
DEPTOR
Low ATP/AMP
AMPK
mTORC1

Figure 1.09 Upstream Regulators of mTORC1. The mTORC1 acts as a central node to sense the status of growth factors, nutrients (glucose and amino acid), stress and energy. Arrows represent activation. Bars represent inhibition. PIP2, phosphatidylinositol-4,5–biphosphate; PIP3, phosphatidylinositol-3,4,5–triphosphate. For the full names of the proteins indicated in this figure, see **Lists of Abbreviations**.

1.5.4.2 Energy regulates mTORC1

In addition to mitogen-stimulated PI3K and Raf/Erk signaling, mTORC1 activity is tightly controlled in response to the extracellular milieu. mTORC1 activity is down-regulated when cells sense low levels of energy, as a result of glucose starvation, by evaluating the decreased ratio of intracellular ATP:AMP. A high intracellular AMP concentration and low ATP concentration promotes interaction between AMP and the γ subunit of AMP-dependent protein kinase (AMPK), which facilitates either liver kinase B1 (LKB1)- or calcium/calmodulin-dependent protein kinase kinase 2, beta (CaMKKβ-dependent phosphorylation of Thr172 on the α subunit of AMPK , resulting in AMPK activation (Oakhill *et al.* 2011). Subsequently, the activated AMPK phosphorylates TSC2 and promotes TSC2-modulated inhibition of Rheb/mTORC1 signaling (Figure 1.09) (Inoki *et al.* 2003).

Alternatively, AMPK inactivates mTORC1 through inhibitory phosphorylation of Raptor (Gwinn *et al.* 2008). Moreover, mTORC1 senses blood glucose status through an AMPK-independent pathway. Under the conditions of glucose starvation, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) interacts with Rheb, which blocks Rheb-mediated mTORC1 activation. Conversely, an adequate supply of glucose prevents GAPDH from binding to Rheb, allowing Rheb to activate mTORC1 (Figure 1.09) (Lee *et al.* 2009).

Another study revealed that p53 tumour suppressor played a role in down-regulation of mTORC1 activity under energy depletion conditions, and that the mechanism involved the activities of AMPK and TSC1/TSC2 complexes (Figure 1.09) (Feng *et al.* 2005).

1.5.4.3 Nutrients regulate mTORC1

The mTORC1 activity is sensitive to amino acid status. Under amino acid deprivation conditions, mTORC1 does not respond to insulin stimulation, suggesting that the availability of amino acids determines the activity of mTORC1 (Hara *et al.* 1998). Although the mechanism of amino acid to modulation of mTORC1 signaling remains to be determined, some proteins have recently been identified as being involved in this amino acid-sensing system. Earlier studies reported the requirement of essential amino acids (EAAs) such as leucine, tyrosine, and phenylalanine for mTORC1 activation (Blommaart *et al.* 1995; Wang *et al.* 1998). However, the mechanism for cells to absorb EAAs remained unclear until a recent study which utilized HeLa and MCF7 cancer cell lines to identify a two-step mechanism responsible for EAAs transportation. The first step is to utilize a solute carrier family 1 member 5 (SLC1A5) transporter to take in L-glutamine (an non-EAA). Following localization of L-glutamine in the cytosol, cells utilize a heterodimeric counter-transporter named solute carrier family 7 member 5 (SLC7A5)/ solute carrier family 3 member 2 (SLC3A2) to pump out L-glutamine and take up L-leucine simultaneously (Figure 1.09) (Nicklin *et al.* 2009). Moreover, accumulating evidence suggests that class III PI3K or human vacuolar protein sorting 34 (hVps34) has a role in activation of mTORC1 signaling in response to both amino acid and glucose stimulation (Byfield *et al.* 2005; Nobukuni *et al.* 2007; Xu *et al.* 2011). It has been shown that an increase in amino acid levels promotes higher intracellular Ca^{2+} concentration, which in turn encourages the interaction between calmodulin (CaM) and class III PI3K, thereby activating mTORC1 signaling (Figure 1.09) (Gulati *et al.* 2008). Another study has reported that class III PI3K acts as a positive regulator of PLD, which functions in the production of phosphatidic acid. Following amino acid stimulation, class

III PI3K-dependent generation of phosphatidic acid can promote the association of mTOR with Raptor (Figure 1.09) (Xu *et al.* 2011).

A recent study of human embryonic kidney (HEK) 293 cells showed that Rag (Ras-related small GTP-binding protein) acted as a positive regulator of mTORC1 activity and promoted translocation of mTOR and Raptor from the cytoplasm to the peri-nuclear region and endomembrane cell compartments, such as ER, in the presence of L-leucine. Moreover, L-leucine stimulation failed to induce mTORC1 activity after down-regulation of Rag expression level. Through indirect evidence, the same study implicated that Rag functioned in promoting Rheb-activated mTORC1 activity at the late endosomal and lysosomal regions (Figure 1.09) (Sancak *et al.* 2008).

Recently, MAP4K3 (mitogen-activated protein kinase (MAPK) kinase kinase kinase 4) was proposed as the link between amino acid and mTORC1, based on the observed up-regulation of MAP4K3 activity and increased phosphorylation of mTORC1 downstream targets p70S6K and 4E-BP1 in response to amino acid stimulation. However, the role of MAP4K3 in regulation of mTORC1 remains unresolved (Figure 1.09) (Findlay *et al.* 2007).

1.5.4.4 Stress regulates mTORC1

It was previously demonstrated that mTORC1 had a role in sensing cellular stress. Recent evidence from primary mouse embryo fibroblasts and *Drosophila* suggested that mammalian REDD1 (regulated in development and DNA damage 1) and Scylla/Charybdis in *Drosophila*, the counterpart of RTP801/REDD1 in mammals, played a key role in the transduction of energy stress signals to the mTORC1 signaling pathway. The expression level of REDD1 was initially increased in the presence of stress inputs such as hypoxia, ATP depletion and ionizing radiation. Subsequently, the increased REDD1 level impaired mTORC1 activity through targeting TSC2 (Figure 1.09) (Reiling and Hafen 2004; Sofer *et al.* 2005). However, more recent findings suggest that stress-mediated mTORC1 inhibition through REDD1 is cell-specific. For

example, in mouse embryonic fibroblasts, hypoxia abrogates mTORC1 signaling through the REDD1/TSC2/Rheb pathway, whereas in primary mouse hepatocytes hypoxia down-regulates mTORC1 activity through a mechanism involved in up-regulation of AMPK and LKB1 activity and Raptor phosphorylation (Figure 1.09) (Wolff *et al.* 2011). An additional study of the impact of mitochondrial dysfunction-mediated osmotic stress on mTOR localization suggested a correlation of stress inputs with redistribution of mTOR at the outer membrane of the mitochondria and suppression of mTORC1-mediated cell growth (Desai *et al.* 2002).

1.5.5 Functions and substrates of mTORC1

1.5.5.1 mTORC1 promotes 4EBP1-regulated cap-dependent translation

mTORC1 inhibits the activity of 4E-BP1 via phosphorylation of four sites, Thr37, Thr46, Thr70 and Ser65, in a hierarchical manner (Mothe-Satney *et al.* 2000). The phosphorylated 4E-BP1 subsequently releases eIF4E, allowing Ser209 on eIF4E to be phosphorylated by MAPK-interacting kinase (Mnk) which is positively regulated by Erk and p38 MAPK. Subsequently, the free and/or phosphorylated eIF4E enters an eIF4F complex which is composed of eIF4A, eIF4E and eIF4G (Figure 1.10). The assembly of the eIF4F complex activates translation of mRNA with 5' terminal cap consisting of m7GpppN (where m7G represents methyl group on the 7^{th} position of guanine nucleotide, ppp represents three phosphate groups and N represents any nucleotide at 5' end of mRNA) (Raught and Gingras 1999).

In vitro studies of a variety of tumour types suggest that Mnk-mediated phosphorylation of eIF4E correlates with proliferation and malignancy of cancer cells, whereas the development of normal cells does not seem to be related to eIF4E activity (Ueda *et al.* 2004; Wang *et al.* 2007; Bianchini *et al.* 2008). The 5'capped mRNAs are further divided into two groups, according to the length of 5' terminal untranslated region (5'UTR). The first group of capped mRNA is characterized by possession of a short 5'UTR, such as 5' terminal oligopolypyrimidine (5'TOP) which encodes ribosomal

proteins, and is usually translated efficiently. Conversely, the second group contains long and secondary structured 5'UTR which is unwound by the eIF4F complex. In normal cells, these capped mRNAs with highly structured 5'UTR are poorly translated, resulting in low expression levels of these proteins (Koromilas *et al.* 1992; Meyuhas and Hornstein 2000 ; Mamane *et al.* 2004). These long 5'UTR capped mRNA usually encode proteins involved in promoting cell cycle progression such as cyclin D1, c-Myc, ornithine decarboxylase (ODC) and $p27^{kip1}$; cell survival such as Bcl-2 and PIM-1; angiogenesis such as VEGF and basic fibroblast growth factor (bFGF-2); and tumour invasion such as matrix metalloprotease 9 (MMP9) (De Benedetti and Graff 2004; Mamane *et al.* 2004). Increasing evidence suggests that eIF4E plays a central role in controlling the translational rate of these long 5'UTR mRNA (De Benedetti *et al.* 1991; Koromilas *et al.* 1992). Up-regulation of eIF4E activity, resulting from either overexpression of eIF4E or deregulation of eIF4E upstream signalling, has been reported to increase translation of these long 5'UTR mRNAs and contribute to cell transformation (Koromilas *et al.* 1992; De Benedetti and Graff 2004).

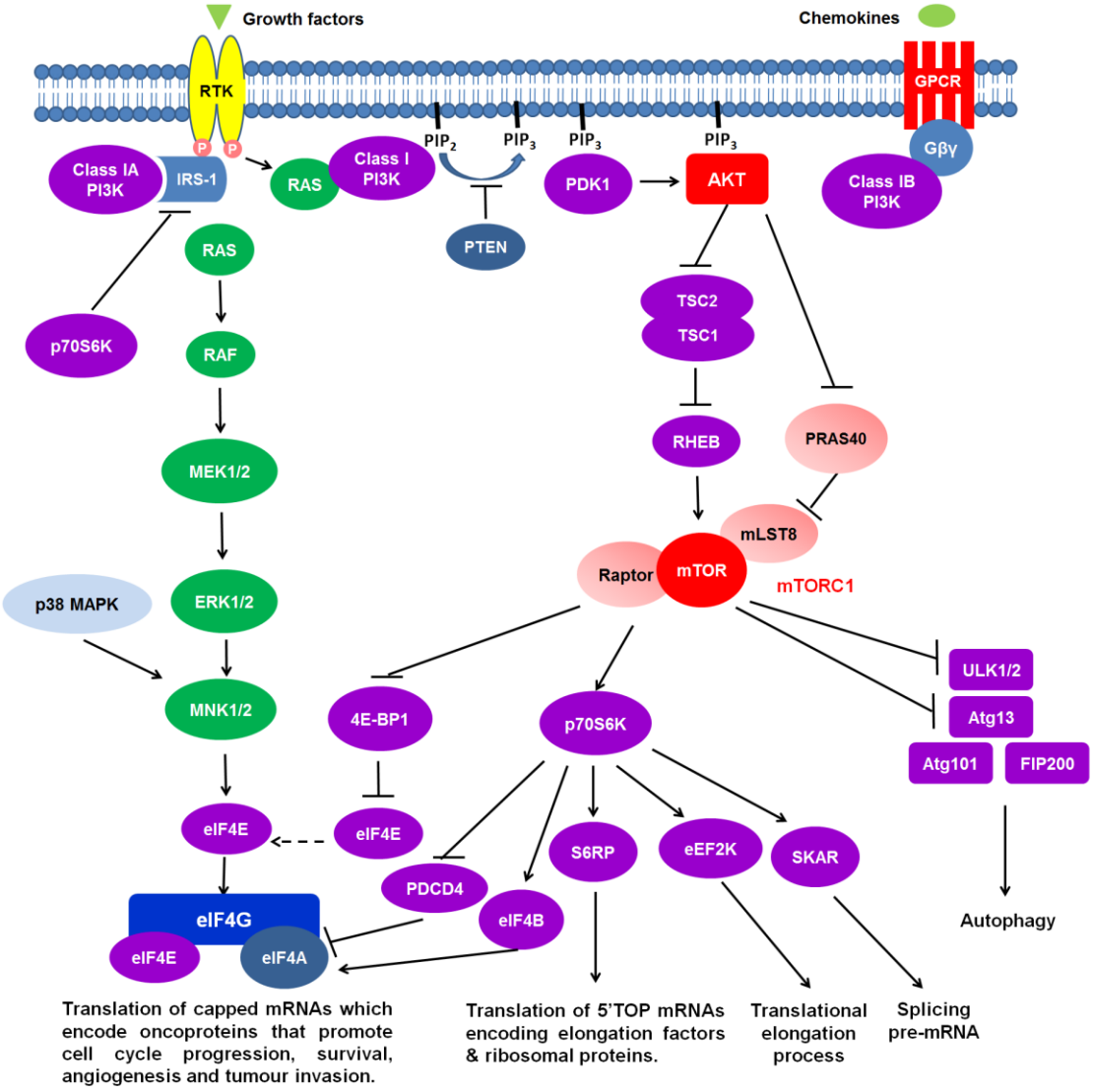


Figure 1.10. Downstream effectors of mTORC1. Activation of mTORC1 results in initiation of protein synthesis and inhibition of autophagy. Notably, translation of capped mRNAs is concurrently regulated by both mTORC1 and Erk/Mnk pathways. Arrows represent activation. Bars represent inhibition. PIP2, phosphatidylinositol-4,5–biphosphate; PIP3, phosphatidylinositol-3,4,5–triphosphate. For the full names of the proteins indicated in this figure, see **Lists of Abbreviations**.

1.5.5.2 mTORC1 promotes p70S6K-regulated translation of 5'TOP mRNA

Meanwhile, the mTORC1-dependent phosphorylation of four serine/threonine residues including Thr229, Thr389, Ser404, and Ser411 on p70S6K drives activation of p70S6K and subsequently stimulates p70S6K-dependent phosphorylation of Ser235, Ser236, Ser240, and Ser244 on its substrate S6RP, resulting in activation of S6RP and triggering translation of 5'TOP mRNA (Figure 1.10) (Ferrari *et al.* 1991; Han *et al.* 1995). The 5'TOP mRNAs encode nearly all ribosomal proteins and elongation factors, and also the e, f, and h subunits of translation initiation factor 3 (eIF3), which implies that the mTOR-activated 5'TOP mRNA translation plays a critical role in controlling rate of translation process (Jefferies *et al.* 1997; Fumagalli and Thomas 2000; Meyuhas and Hornstein 2000 ; Iadevaia *et al.* 2008).

1.5.5.3 Other functions of mTORC1/p70S6K signaling

In addition to S6RP, activated p70S6K has been found to accelerate mRNA translation machinery through direct phosphorylation of other protein substrates such as eukaryotic eEF2K, eIF4B, and programmed cell death 4 (PDCD4) (Figure 1.10) (Wang *et al.* 2001; Raught *et al.* 2004; Dorrello *et al.* 2006). For example, p70S6K stimulates eukaryotic elongation factor 2 (eEF2)-mediated elongation during translation process through inhibitory phosphorylation of eEF2 kinase, which acts as a negative regulator of eEF2 (Wang *et al.* 2001). The p70S6K can enhance helicase activity of eIF4A, which is a member of eIF4F complex, through positive regulation of eIF4B and negative regulation of PDCD4 (Raught *et al.* 2004; Dorrello *et al.* 2006). Additionally, recent findings showed that active p70S6K was recruited by SKAR onto newly synthesized mRNAs and functioned in splicing these pre-mRNAs, thereby accelerating mRNA translation machinery following mTORC1-mediated activation of p70S6K (Ma *et al.* 2008).

Although active mTORC1/p70S6K signaling enhances mRNA translation efficiency, *in vitro* and *in vivo* studies of adipocytes and muscle cells have shown that

activation of p70S6K induces inhibitory phosphorylation of Ser636/639/1101 on IRS-1, which impairs insulin-stimulated class I PI3K activity (Figure 1.10). The purpose of the p70S6K-induced negative feedback loop is to maintain glucose homeostasis. Recently, this negative feedback has been found to be linked to insulin resistance and diabetes mellitus (Tremblay *et al.* 2007; Veilleux *et al.* 2010). Moreover, this negative feedback loop was associated with resistant mechanism of cancer therapy that targets class I PI3K signaling (Carracedo *et al.* 2008; Chen *et al.* 2010).

1.5.5.4 mTORC1 negatively regulates autophagy

Recently, evidence suggests that mTORC1 plays a role in negatively regulating autophagy through phosphorylation of Unc-51 like kinase 1 (ULK1)/ULK2 and autophagy-related 13 (Atg13) (Figure 1.10) (Yan *et al.* 1999). Autophagy involves the formation of a double-membraned autophagosome to enwrap proteins, organelles and even pathogens, followed by fusion of the autophagosome and a lysosome, allowing hydrolases in the lysosome to digest the contents. Under nutrient deprivation or energy stress conditions, cells may undergo autophagy in order to efficiently utilize limited energy sources and maintain cell survival (He and Klionsky 2009). In mammalian cells, insufficient nutrient supplies or the presence of energy stress down-regulates mTORC1-dependent phosphorylation of ULK1/2 and Atg13, which in turn promotes ULK1/2 auto-phosphorylation and induces ULK1/2 to phosphorylate Atg13 and FIP200 (focal adhesion kinase (FAK) family interacting protein of 200 kDa), resulting in formation of an ULK1/2-Atg13-FIP200 complex-regulated autophagosome (Yan *et al.* 1999).

1.6 Dysregulation of class I PI3K/Akt/mTOR axis pathway in tumours

1.6.1 Molecular alterations of components of class I PI3K/Akt/mTOR signaling

Over the past two decades, a large body of literature has shown that over expression of the class I PI3K/Akt/mTOR pathway is tightly correlated with

tumourigenesis. The constitutive activation of PI3K kinase cascades can be instigated by molecular alterations in any component of the class I PI3K/Akt/mTOR axis pathway and its upstream signals. With regard to class I PI3K, amplification (or multiple gene copies) and somatic mutations of *PIK3CA* which encodes the p110α catalytic subunit (Samuels *et al.* 2004; Pedrero *et al.* 2005), amplification of *PIK3CB* encoding the p110β catalytic subunit (Liu *et al.* 2008), over-expression of *PIK3CD* mRNA without amplification of *PIK3CD* which encodes the p110δ catalytic subunit (Knobbe and Reifenberger 2003), and somatic mutations of *PIK3R1* encoding the p85α regulatory subunit (Philp *et al.* 2001) have been reported. Loss or attenuation of PTEN function was mostly attributed to loss of heterozygosity (LOH), somatic mutations of *PTEN* or both (Chiariello *et al.* 1998; Sakai *et al.* 1998; Byun *et al.* 2003; Knobbe and Reifenberger 2003). Methylation of *PTEN* promoter resulting in loss of PTEN expression has been described in human cervical cancer (Cheung *et al.* 2004). PTEN activity can be suppressed through post-translational modification. A study of T cell leukemia showed that either up-regulation of casein kinase 2 (CK2) activity or elevation of reactive oxygen species (ROSs) levels promoted phosphorylation of serine/threonine residues at C-terminus on PTEN, resulting in attenuation of PTEN activity (Silva *et al.* 2008). Although PDK1 was not considered to be oncogenic, amplification of *PDPK1* which resulted in over expression of PDK1 mRNA and protein was found to enhance effects of aberrant expression of PDK1 upstream signaling (Maurer *et al.* 2009).

Alterations of three Akt isoforms, including amplification of *Akt1*, somatic (activating) mutations of *Akt1*, amplification of *Akt2*, overexpression of *Akt2* without evidence of *Akt2* amplification, amplification of *Akt3*, overexpression of *Akt3* mRNA and protein but lack evidence of *Akt3* amplification, and somatic (activating) mutations of *Akt3*, have been reported in a wide range of tumour types (Bellacosa *et al.* 1995; Nakatani *et al.* 1999; Knobbe and Reifenberger 2003; Carpten *et al.* 2007; Davies *et al.* 2008; Kim *et al.* 2008; Kirkegaard *et al.* 2010). Recently, deletion of either *Akt1* or *Akt2* has been detected in 4.8% and 21% of breast carcinomas respectively by utilizing fluorescence in situ hybridization (FISH). However, further study of the effect of Akt

gene deletion was not carried out (Kirkegaard *et al.* 2010). It was previously demonstrated that overexpression of Akt2 was responsible for tumourigenicity and invasion behavior by comparison of tumour-initiation capability of human pancreatic cancer PANC1 cells exhibiting high levels of Akt2 expression with PANC1 cells transfected with antisense *Akt2* RNA in a nude mouse model (Cheng *et al.* 1996). In contrast to *Akt2*, expression of wild-type *Akt1* was unable to induce cell transformation. However, NIH3T3 fibroblasts transfected with Myr-Akt1 construct for constitutive activation of Akt1 kinase were found to induce tumourigenesis in nude mice (Sun *et al.* 2001). To date, there are controversial studies on the role of Akt3 in cancer development. In cell lines derived from estrogen-negative breast cancers or androgen-insensitive prostate carcinomas, overexpression of Akt3 tightly correlated with increased Akt3 kinase activity and was associated with tumour aggressiveness, whereas expression of Akt3 inversely correlated with malignant phenotype of gliomas (Kirkegaard *et al.* 2010; Mure *et al.* 2010).

Somatic mutations or LOH in either TSC1 or TSC2 tumour suppressor were reported to cause inactivation of TSC1-TSC2 complex and frequently gave rise to benign tumours such as lymphangioleiomyomatosis and hamartomas. Sporadically, these benign tumours developed to malignant phenotypes such as renal cell carcinomas. Accumulating evidence suggests that loss of TSC1/TSC2 function not only caused high levels of mTORC1 signaling but also released TSC1/TSC2-mediated inhibition of Wnt/β-catenin signaling and transforming growth factor beta (TGFβ)-dependent growth inhibition (Niida *et al.* 2001; Mak and Yeung 2004; Rendtorff *et al.* 2005).

So far, only one recent study has described a point (activating) mutation in the kinase domain of yeast *TOR2*, the mammalian homologue mTOR. However, it has been found that mutant mTOR introduced into NIH/3T3 fibroblasts did not transform cells (Ohne *et al.* 2008). Molecular alterations of mTORC1 downstream effectors including amplification and subsequent overexpression of *RPS6KB1* encoding S6K1, or *EIF4E* encoding eIF4E, have been detected in breast cancers and head and neck squamous cell carcinomas (Sorrells *et al.* 1999; Heinonen *et al.* 2008). Interestingly, although 4EBP1

functions in blockade of eIF4E-mediated cap-dependent translation, two recent studies of breast cancers suggests that amplification or overexpression of 4EBP1 is associated with malignant behavior and a poor prognosis (Braunstein *et al.* 2007; Karlsson *et al.* 2010). In experiments with breast cancer cell lines and a chick embryo model, concomitant overexpression of 4EBP1 and eIF4G under hypoxic conditions was shown to promote tumour survival and angiogenesis through up-regulation of translation of mRNAs containing internal ribosome entry sites (IRESs), but not through promoting cap-dependent translation machinery (Braunstein *et al.* 2007).

1.6.2 Molecular alterations of upstream inputs of class I PI3K

Recently, many studies have shown that constitutive activation of class I PI3K kinase cascades can be as a result of alterations of its upstream inputs, such as RTKs, tyrosine kinases and Ras. So far, activating mutations in several RTKs, such as KIT, FMS-like tyrosine kinase 3 (Flt3), EGFR and fibroblast growth factor receptor (FGFR), have been identified in a variety of cancers. For instance, a high frequency of D816V mutation in kinase domain and V560G mutation in juxtamembrane domain in *c-kit*, which encodes KIT protein, was reported in human systemic mastocytosis (SM) and gastrointestinal stromal tumors (GISTs) respectively. These point mutations were found to enable constitutive activation of KIT via phosphorylation of tyrosine residues despite the absence of stem cell factor (SCF) ligand (Furitsu *et al.* 1993; Hirota *et al.* 1998). Similarly, activating mutations, encompassing tandem duplications, deletions, transitions, and insertions, in the juxtamembrane domain of *c-kit* have been identified in canine mast cell tumours (London *et al.* 1999; Ma *et al.* 1999). In acute myeloid leukemia (AML), either internal tandem duplication or activation loop mutation in *FLT3* has been found to induce constitutive activation of FLT3-mediated MAPK, STAT and class I PI3K pathways (reviewed in (Meshinchi and Appelbaum 2009)). High frequency of amplification of *EGFR variant III* (*EGFRvIII*), which is characterized by a deletion from exon 2 to exon 7 and subsequent fusion of exon 1 and exon 8 with creation of a glycine codon between the two exons in the extracellular domain of *EGFR*, has been

reported to cause overexpression and constitutive activation of this mutant EGFR in glioblastoma multiforme (GBM) (Humphrey *et al.* 1988; Sugawa *et al.* 1990). Other mutant EGFRs resulting in ligand-independent activation of this RTK include EGFRvI which consists of an N-terminal truncation in the extracellular domain, double mutant EGFRvIII/Δ12-13 which contains a deletion from exon 2 to7 and exon 12 to 13 in the extracellular domain, EGFR.TDM/18–25 and EGFR.TDM/18–26 which contain intracellular tandem duplication of exon 18 to 25 and exon 18 to 26 respectively (Wong *et al.* 1992; Callaghan *et al.* 1993; Ciesielski and Fenstermaker 2000). Translocation of the chromosome containing the *FGFR1* gene, leading to fusion of *FGFR1* with other genes, was commonly found in 8p12 stem cell myeloproliferative disorder (MPD), characterized by myeloid hyperplasia, eosinophilia and lymphoblastic lymphoma. So far, *FGFR1* has been reported to be translocated and fused with *FIM*, *FOP*, *CEP110*, *BCR*, or *HERV* gene, resulting in constitutive activation of FGFR1 signaling in MPD (Popovici *et al.* 1998; Popovici *et al.* 1999; Guasch *et al.* 2000; Fioretos *et al.* 2001; Guasch *et al.* 2003). Aberrant expression of fusion tyrosine protein BCR (breakpoint cluster region)-ABL (Abelson) as a result of reciprocal translocation of chromosomes 9 and 22, and generation of a chimeric *BCR-ABL* gene on chromosome 22, has been found in more than 90% of patients with chronic myelogenous leukemia (CML) and a minority group of patients with either acute lymphoblastic leukemia (ALL) or AML. The cytoplasmic BCR-ABL fusion protein is a constitutively activated tyrosine kinase that induced cell transformation through triggering Jak-Stat, class I PI3K/Akt, and Ras pathways and blocked apoptosis (Reviewed in (Skorski *et al.* 1997; Kurzrock *et al.* 2003)). Point (activating) mutations of three isoforms of oncogenic *Ras*, comprising *H-Ras*, *K-Ras*, and *N-Ras* genes, have been reported in a wide range of human cancers and canine lung tumours (http://www.sanger.ac.uk/genetics/CGP/cosmic/) (Richter *et al.* 2005; Castellano and Downward 2011; Fernandez-Medarde and Santos 2011; Forbes *et al.* 2011). For instance, previous studies have shown point mutations of *K-ras* in 95% of human pancreatic carcinomas (Almoguera *et al.* 1988). *K-ras* mutations were also detected in 16.4% of human and 25% of canine non-small cell lung cancer (NSCLC) specimens (Kraegel *et al.* 1992; Graziano *et al.* 1999). It has been suggested that *N-ras*

mutations are more related to early onset of primary cutaneous melanoma than metastasis of melanoma, and the frequency of *N-ras* mutations in primary melanoma is 28% (Omholt *et al.* 2002). Mutations of the *H-ras* gene have been observed in 9 out of 30 (30%) human transitional cell carcinoma specimens. Moreover, comparison of these 30 bladder cancer specimens with the adjacent normal tissue detected a high incidence (77%) of overexpression of one or more *Ras* genes (Boulalas *et al.* 2009). Although the predominant downstream signaling of Ras is often referred to as the Raf/Mek/Erk pathway, constant signal transduction of mutant Ras has been demonstrated to be, in part, via interaction with the p110α and/or p110β subunits of class I PI3K (Moodie *et al.* 1993; Warne *et al.* 1993; Lim and Counter 2005; Gupta *et al.* 2007; Jia *et al.* 2008).

1.6.3 Molecular alterations of mTORC2

So far, little information regarding mTORC2 dysfunction is available. However, one study of glioma has reported that mTORC2 activity, and its regulation of invasive behavior, were increased as a result of the up-regulation of Rictor mRNA and protein (Masri *et al.* 2007).

1.7 Targeting the class I PI3K/Akt/mTOR pathways for cancer therapy

1.7.1 mTOR inhibitors

The class I PI3K/Akt/mTOR signaling pathway regulates many functions including cellular growth, proliferation, angiogenesis, metabolism and motility, all of which are critical for normal cell development and tumourigensis. Drugs that specifically target this pathway have been developed to treat cancer and have entered clinical trials. Among these class I PI3K/Akt/mTOR inhibitors, Rapamycin (also known as Sirolimus), which is a product of the bacterium *Streptomyces hygroscopicus*, is the first indentified compound that specifically targets TOR in yeast and mTOR in mammals. Rapamycin acts as a cytostatic to accumulate cells at G0/G1 phase (Huang *et*

al. 2003). The mechanism by which Rapamycin blocks mTOR signaling involves two steps; Rapamycin interacts with FKBP-12, a cytosolic receptor, resulting in a Rapamycin-FKBP12 complex which then binds to an FRB domain in the mTOR protein, abolishing its downstream activities (Figure 1.06) (Huang and Houghton 2003). However, Rapamycin is no longer used to treat human patients with cancer due to its potent immunosuppression and poor pharmacological properties, such as water insolubility and instability (Huang *et al.* 2003). Nevertheless, it has recently been demonstrated to safely treat canine patients with osteosarcoma and a clinical trial in canine oncology is currently ongoing (Paoloni *et al.* 2010). In human cancer studies, three novel Rapamycin analogues or Rapalogues, Temsirolimus (also known as CCI-779), Everolimus (also known as RAD001) and Ridaforolimus (also known as AP23573 or MK-8669), are being investigated in clinical trials which are either ongoing or recently completed. These three mTOR inhibitors, which exhibit different pharmacokinetic properties, have similar mechanisms towards the blockade of mTOR activity, with the advantage of improved aqueous solubility relative to their parent compound Rapamycin (Yuan *et al.* 2009). For instance, Temsirolimus displays improved aqueous solubility for intravenous use and no observable immunosuppression, whereas Everolimus is orally bioavailable and displays immunosuppressive activity (Alexandre *et al.* 1999; Boulay *et al.* 2004). Ridaforolimus can be administered orally or intravenously and is an immunosuppressant (Mita *et al.* 2008).

With regard to the clinical trials of these Rapalogues, the most promising results with Temsirolimus and Everolimus have been observed in patients with advanced or relapsed RCC, and both inhibitors have been approved by U.S. Food and Drug Administration (FDA) for the treatment of advanced renal cell carcinoma (RCC) (Hudes *et al.* 2007; Wang 2010). Although some adverse reactions (e.g. skin symptoms) and abnormalities of the blood and serum profile (e.g. hyperglycemia, anemia) were commonly seen in patients treated with Temsirolimus in phase III clinical trials, severe reactions such as interstitial pneumonia rarely occurred. The median survival time in metastatic RCC patients receiving Temsirolimus alone (10.9 months) was longer than

those receiving interferon alone (7.3 months) or in combination with Temsirolimus (8.4 months) (Hudes *et al.* 2007; Kwitkowski *et al.* 2010). In a phase III trial of Everolimus in patients with metastatic RCC after failure of treatment with vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors (TKIs), the median progression-free survival in Everolimus and placebo groups were ~5 months and 1.9 months respectively. The overall survival in the Everolimus group was 1.9-fold longer than that of the placebo group (Motzer *et al.* 2010). The most common adverse effects related to Everolimus were stomatitis and a rash, less than 5% of patients developed severe infections or symptoms (Motzer *et al.* 2008; Motzer *et al.* 2010). In 2011, Everolimus was approved by the FDA for the treatment of advanced pancreatic neuroendocrine tumours (pNET), based on the promising results obtained from a phase III trial of Everolimus in patients with advanced pNET after failure of radiation therapy (Saif 2011; Yao *et al.* 2011). This trial showed that the median progression-free survival was 11 months in the Everolimus group, which was approximately 6 months longer than in the placebo group. In addition, 34% of patients treated with Everolimus were progression-free at 18 months or longer, whereas only 9% of patients treated with placebo achieved the same result (Yao *et al.* 2011). Although Ridaforolimus is not yet approved for the treatment of any cancer type, the most promising results with this inhibitor have been obtained in soft tissue and bone sarcomas (Mita *et al.* 2008). Currently, intravenous and oral formulations of Ridaforolimus are being investigated in a phase III trial in patients with sarcomas and the scheme is entitled "Sarcoma mUlti-Center Clinical Evaluation of the Efficacy of riDaforolimus" (SUCCEED). Ridaforolimus was reported to be well-tolerated in patients with a variety of cancers and the most common side effects, such as mucositis, mouth sores, fatigue and myelosuppression, were mild and generally reversible (Mita *et al.* 2008; Rizzieri *et al.* 2008; Hartford *et al.* 2009).

Despite the efficacy of these mTOR inhibitors in treating advanced RCC, pNET and certain sarcomas, other tumour types showed lower responses (Yuan *et al.* 2009). For instance, the results obtained from a phase II trial in mantle cell lymphoma (MCL)

were once considered promising; this trial showed that 35 patients with relapsed/refractory MCL whose disease had progressed on chemotherapy or the monoclonal antibody therapy Rituximab had a 38% overall response rate with a median disease progression-free in all patients of 6~7 months (Witzig *et al.* 2005). In a phase III trial for the treatment of advanced MCL, the median progression-free survival (PFS) in the patients receving either one of two regimes of Temsirolimus monotherapy, 175/75-milligram (mg) and 175/25-mg, was 4.8 and 3.4 months respectively, whereas the median PFS in the patients receiving investigator's choice treatment was 1.9 months. The Temsirolimus (175/75-mg) group had longer median overall survival (12.8 months) and a higher objective response rate (22%) compared with the investigator's choice (9.7 months and 2%) (Hess *et al.* 2009). However, due to the utilization of high doses of Temsirolimus in this study, severe adverse events were observed, such as haematological abnormalities in the majority of patients, grade 3 or 4 thrombocytopenia in 50-60% of patients and death in 5 of the patients. The limited efficacy but high risk of severe drug-related side effects limits the use of Temsirolimus as a single agent in advanced MCL (Hess *et al.* 2009).

Recently, novel chemical compounds which specifically target both mTORC1 and mTORC2 have been developed. PP242 was designed to inhibit both mTOR complexes by targeting an ATP binding site in the catalytic domain of mTOR kinase. It has been demonstrated that the efficacy of PP242 in inhibiting mTORC1-mediated downstream targets is much better than Rapamycin, as evidenced by more potent inhibition of 4EBP1 phosphorylation and cap-dependent translation compared to Rapamycin. PP242 also inhibited cellular proliferation more potently than Rapamycin. However, the inhibitory effect of PP242 on Akt substrates (through mTORC2/Akt inhibition) was modest. Moreover, PP242 developed autophagic resistance mechanism which counteracted drug efficacy more significantly than Rapamycin (Feldman *et al.* 2009). An *in vivo* study of acute leukemia initiated by overexpression of BCR-ABL tyrosine kinase showed that the efficacy of PP242 in leukemia treatment was greater than that of Rapamycin, and PP242 was less toxic to normal lymphocytes compared to

Rapamycin (Janes *et al.* 2010). AZD8055 is another novel ATP-competitive inhibitor targeting both mTOR complexes, and is orally bioavailable. The effects of AZD8055 on inhibition of both mTORC1 and mTORC2, and induction of autophagy resistance mechanism to counteract drug efficacy, were similar to PP242 both *in vitro* and *in vivo*. In a mouse model of tumour xenografts, no drug-related cytotoxicity was observed in the AZD8055 treatment group. AZD8055 is currently being investigated in a phase I clinical trial in patients with glioblastoma multiforme (GBM), advanced hepatocellular carcinoma, and other advanced solid tumours (Chresta *et al.* 2010).

1.7.2 Class I PI3K inhibitors

With regard to inhibitors targeting class I PI3K, early studies showed that LY294002, a quercetin compound, had a higher specificity of inhibition for all isoforms of class I PI3K and CK2 than other protein kinases, and effectively inhibited tumour growth in a mouse model (Sanchez-Margalet *et al.* 1994; Davies *et al.* 2000; Hu *et al.* 2000). Another early identified pan-class I PI3K inhibitor is Wortmannin, which is a product of the fungus *Penicillium wortmannii* (Powis *et al.* 1994). Unlike LY294002 which inhibits class I PI3K in an ATP-competitive manner, Wortmannin irreversibly inhibits PI3K activity by binding covalently to Lys802, which is a residue in the catalytic domain of p110α and responsible for transfering a phosphate group from ATP to substrate proteins (Wymann *et al.* 1996). Wortmannin was reported to have anti-tumour activity against tumour xenografts in a mouse model (Schultz *et al.* 1995). However, due to the dermatological toxicity of LY294002, the liver and hematological toxicity of Wortmannin, and the poor solubility and other phamacological disadvantages of both drugs, the usage of LY294002 and Wortmannin is confined to laboratory studies (Schultz *et al.* 1995; Hu *et al.* 2000). To overcome the poor pharmacological properties and significant side effects of Wortmannin, a novel Wortmannin analogue, PX-866, was synthesized with improved pharmacological properties compared with Wortmannin, such as longer drug duration, higher stability and less cytotoxicity (Ihle *et al.* 2004). Currently, PX-866 has entered phase I clinical trials for drug toxicity in healthy

volunteers and cancer patients, the results of which are pending (clinical trials identifiers (IDs): NCT00726583 and NCT01408316; http://clinicaltrials.gov/ct2/search). In addition to PX-866, another novel pan-class I PI3K inhibitor that reversibily abolishes PI3K activity in an ATP-competitive manner is ZSTK474, an s-triazine derivative compound. An *in vivo* study has shown that ZSTK474 had anti-tumour effects in a tumour xenograft mouse model, and no severe organ toxicity was observed (Yaguchi *et al.* 2006). Currently, a stage I trial of the phamarcological safety of ZSTK474 in human patients is ongoing, and the results are pending (clinical trial ID: NCT01280487; http://clinicaltrials.gov/ct2/search). The third newly synthesized pan-class I PI3K inhibitor is GDC-0941 which shares similar inhibition profile for all classes of PI3Ks with ZSTK474 (Kong *et al.* 2010). GDC-0941, a thieno[3,2-d]pyrimidines derivative, is orally bioavailable and has entered phase I clinical trials to evaluate drug safety in cancer patients (clinical trial IDs: NCT00999128 and NCT01474668; http://clinicaltrials.gov/ct2/search).

There are also newly developed inhibitors that specifically target either one or two isoforms of class I PI3K. For example, TGX-221, a LY294002 analogue, was developed to selectively inhibit class I PI3Kβ-induced adhesion of integrin α(IIb)β(3) and thereby prevent platelets aggregation and thrombus formation (Jackson *et al.* 2005). AS-605240, a selective class I PI3Kγ inhibitor, was reported to suppress PI3Kγ-induced inflammation in mose model of systemic lupus erythematosus (SLE) (Barber *et al.* 2005). TG100-115 was designed to specifically target class I PI3Kγ and δ, thereby eliminating PI3Kγ- and δ-induced inflammation and edema (Doukas *et al.* 2006).

1.7.3 Dual Class I PI3K and mTOR inhibitors

Recently, inhibitors targeting class I PI3K, mTORC1 and mTORC2, such as PI103 and NVP-BEZ235, have been developed and entered clinical trials. For example, NVP-BEZ235 is an orally bioavailable inhibitor which targets ATP binding sites in the catalytic domains of these enzymes. NVP-BEZ235 has displayed effective inhibition of cell proliferation and induction of apoptosis *in vitro* (Maira *et al.* 2008; Baumann *et al.*

2009). This dual class I PI3K/mTOR kinase inhibitor was also found to be well tolerated *in vivo*, and exhibited potent anti-tumour activity against human tumour xenografts in a mouse model (Maira *et al.* 2008). Despite the high efficacy of NVP-BEZ235 in inducing apoptosis, this compound was found to significantly induce autophagic resistance. It has therefore been suggested that it be used in combination with the autophagy inhibitor chloroquine (CQ) (Xu *et al.* 2011). NVP-BEZ235 is currently being investigated in phase I/II clinical trials in patients with advanced solid malignancies (ID: NCT00620594), advanced RCC (ID: NCT01453595), or advanced endometrial carcinoma (ID: NCT01290406) (http://clinicaltrials.gov/ct2/search) (Maira *et al.* 2008).

1.7.4 Akt inhibitors

To date, several novel inhibitors which selectively target all isoforms of Akt kinases have entered phase I clinical trials in cancer patients. GSK690693, an ATP-competitive pan-Akt inhibitor, was shown to suppress proliferation and induce apoptosis of 50% of cell lines derived from hematological malignancy whereas normal T lymphocytes were not affected by this inhibitor (Levy *et al.* 2009). GSK690693 showed significant anti-tumour activity against xenograft tumours in a mouse model. However it also caused drug-associated acute hyperglycemia due to inhibition of Akt-mediated glucose uptake (Rhodes *et al.* 2008). So far, an ATP-competitive inhibitor designed to specifically target Akt kinase has not been identified due to the high similarity in ATP-binding pockets between Akt and some of AGC kinase family (a protein kinase group is named after cAMP-dependent protein kinase 1 (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C (PKC)) (Cheng *et al.* 2005; Rhodes *et al.* 2008). Akt inhibitor VIII is an allosteric Akt inhibitor that does not compete with ATP for binding to the catalytic (kinase) domain of Akt. Instead, Akt inhibitor VIII binds Trp80 residue located in PH domain of cytosolic Akt kinase, resulting in maintenance of Akt in a closed and inactive conformation, preventing Akt membrane recruitment by PtdIns(3,4,5)P_3 and hindering PDK1-mediated phosphorylation of Thr308. In addition, the association of inhibitor VIII with PH domain of Akt results in incorrect positioning

of the c-terminal hydrophobic motif of Akt, where residue Ser473 is located. As a result, Akt inhibitor VIII impairs mTORC2-modulated phosphorylation of Ser473 on Akt. However, the allosteric inhibitor VIII-mediated anti-tumour activity is PH domain-dependent. Moreover, due to slight differences in the closed conformation between Akt3 and the other two Akt isoforms (Akt 1 and Akt2), Akt3 is insensitive to this inhibitor (Calleja *et al.* 2009). A modified Akt inhibitor VIII, MK206, has been investigated in monotherapy or combination therapy against tumour activity *in vitro* and *in vivo*; the results showed that MK-2206 had a high efficacy in tumours harboring mutiations of class I PI3K/Akt pathway, but had moderate or no efficacy in tumours with mutations of other pathways (Hirai *et al.* 2010; Meng *et al.* 2010; Liu *et al.* 2011). MK-2206 has been reported to synergize with receptor tyrosine kinase inhibitors, chemotherapeutic inhibitors or a MEK inhibitor when used to treat a variety of tumours *in vitro* and *in vivo* (Hirai *et al.* 2010; Meng *et al.* 2010). To date, preclinical studies and phase I clinical trials have reported MK-2206 as being well-tolerated in patients and animal models (Lindsley 2010). Perifosine is a novel oral alkylphospholipid and its structure resembles lysophosphatidylcholines (LPC), a component of lipid bilayer cell membrane. The mechanism by which Perifosine inhibits Akt kinase is twofold; by directly binding the PH domain of Akt, and by utilizing its long hydrocarbon chain to anchor the phospholipid membrane and accumulate lipid rafts composed of phospholipid bilayer and protein receptors. As a result, the association of Akt with Perifosine prevents Akt from being recruited by PtdIns(3,4,5)P_3. Currently, Perifosine is under investigation in phase II clinical trials which are confined to patients affected by chondrosarcoma, alveolar soft tissue sarcoma or Waldenström's macroglobulinemia (WM) (Gills and Dennis 2009). Of the 37 patients with relapsed/refractory WM, 13 showed partial or minimal responses (35%), 20 showed stable disease (54%), and the median progression-free survival was approximately one year (Ghobrial *et al.* 2010). The most frequent adverse effects were gastrointestinal symptoms (Argiris *et al.* 2006; Ghobrial *et al.* 2010). However, Perifosine was observed to be well-tolerated in patients with WM (Ghobrial *et al.* 2010). Most phase II trials of Perifosine monotherapy in patients with other types of tumour have been suspended due to the disappointing results of phase I or

II trials (Argiris *et al.* 2006; Gills and Dennis 2009). A previous *in vivo* study suggested that the efficacy of Perifosine for cancer treatment is correlated with the degree of Akt inactivation present (Hennessy *et al.* 2007). However, lack of data on biomarkers such as Akt phosphorylation in specimens from patients in these trials limited the interpretation of the results in this respect. In preclinical studies, Perifosine was shown to inhibit Akt kinase activity and down-regulate Thr308 and Ser473 phosphorylation without affecting the activity of PDK1, mTORC2 and PI3K (Kondapaka *et al.* 2003; Papa *et al.* 2008). In addition to Akt inhibition, Perifosine has been reported to induce apoptosis or cell cycle arrest through modulation of other pathways, such as up-regulation of $p21^{WAF1}$ expression, down-regulation of Erk phosphorylation, stimulation of p38 and Jun N-terminal kinase (Jnk), etc (Patel *et al.* 2002; Kondapaka *et al.* 2003; Papa *et al.* 2008). Triciribine or API-2 (Akt/PKB signaling inhibitor-2), a purine analogue compound, was identified to function in inhibition of DNA synthesis and was used as a chemotherapeutic agent in phase I and II clinical trials for treatment of a variety of cancers (Cobb *et al.* 1983; Mittelman *et al.* 1983; Wotring *et al.* 1990; Hoffman *et al.* 1996). However, due to the requirement for high doses to achieve significant efficacy, leading to the presence of the drug-related side effects, Triciribine is currently no longer used as cytotoxic agent in the clinic setting (Cheng *et al.* 2005). Since 2004, Triciribine has been identified as a highly specific Akt inhibitor that has potent efficacy in tumours harbouring over-expression of Akt signaling *in vitro* and *in vivo*. No side effects were observed in a mouse model when used at 1 mg/ kilogram (kg) per day (Yang *et al.* 2004). The mechanism by which Triciribine inhibits Akt activity is by binding the PH domain of Akt, thereby preventing Akt being recruited by PtdIns(3,4,5)P_3 to inner side of the cytoplasm and subsequently activated by PDK1 (Berndt *et al.* 2010). Currently, a phase I trial of Triciribine in patients with metastatic cancer has been completed but the results have not been published yet (clinical trial IDs: NCT00363454; http://clinicaltrials.gov/ct2/search).

A novel synthetic compound named KP372-1 was originally identified as an inhibitor that selectively targeted Akt (Mandal *et al.* 2005; Mandal *et al.* 2006). Soon

after, this inhibitor was found to have two additional targets, PDK1 and Flt3 (Zeng *et al.* 2006). To date, the mechanism by which KP372-1 inhibits Akt activity remains unresolved. *In vitro* studies have shown that KP372-1 can effectively inhibit the proliferation of tumour cells without affecting the growth of normal cells, and the anti-tumour activity of KP372-1 was found to preferentially induce apoptosis (Koul *et al.* 2006; Zeng *et al.* 2006). So far, no *in vivo* data regarding KP372-1 has been published yet.

1.7.5 Class I PI3K pathway inhibitors in combination with chemotherapeutic agents

Accumulating evidence implies that constitutive activation of the class I PI3K pathway contributes to chemoresistance in cancer due to up-regulation of PI3K-mediated survival, cell cycle progression and protein synthesis (Brognard *et al.* 2001; Clark *et al.* 2002; Hu *et al.* 2002; Lee *et al.* 2004). For instance, Doxorubicin, a chemotherapeutic agent, was reported to stimulate Akt survival factor in breast cancer cell lines for up to 24 hours (Clark *et al.* 2002). Another study of NSCLC cell lines showed that the pan-class I PI3K inhibitor LY294002 enhanced the efficacy of chemotherapy in cells expressing high Akt activity, but this inhibitor did not potentiate chemotherapy-mediated anti-tumour activity in cells with low Akt activity. Furthermore, after introducing constitutively active Akt into the cells exhibiting low Akt activity, the efficacy of chemotherapy was attenuated (Brognard *et al.* 2001). In some prostate cancer cell lines increased expression levels of multidrug resistance protein-1 (MRP-1) and breast cancer resistance protein (BCRP), which function in pumping out chemotherapeutic agents, were correlated with overexpression of class I PI3K activity (Lee *et al.* 2004; Sherbakova *et al.* 2008). Based on the findings mentioned above, *in vitro* and *in vivo* experiments of chemotherapy in combination with class I PI3K pathway inhibitors such as LY294002, Akt inhibitor I, Everolimus and NVP-BEZ235 were conducted, and most of the results showed that inhibition of the PI3K pathway did

sensitize tumour cells to chemotherapy (Brognard *et al.* 2001; Clark *et al.* 2002; Hu *et al.* 2002; Lee *et al.* 2004; Wang *et al.* 2009; Manara *et al.* 2010; O'Reilly *et al.* 2011).

1.8 Cancer stem cell (CSC) theory

1.8.1 CSC theory

Traditionally, Cancer Disease is thought of as cells with accumulation of mutations and each cell within a tumour has an equal opportunity to acquire the abilities of unlimited replication in an uncontrolled manner, escape apoptosis and malignant behaviours such as angiogenesis, invasion and metastasis, resulting in the enlargement of tumour mass and tumour progression. To date, most cancer therapies used in clinics are based on this concept. Although most benign tumours and low grade cancers can be cured, high grades cancers such as advanced renal cell carcinomas often respond to these therapies during a short period of time but relapse later (Withrow and Vail 2007). Over the past three decades, growing evidence has suggested that a single cancer is heterogeneous and contains subpopulations of cells with various biological properties, such as cellular morphology, karyotype, antigen expression, gene expression, response to chemotherapy and radiation therapy, etc (Heppner 1984). For example, multiple subpopulations of cells with diverse metastatic proclivity were isolated from single murine tumours (Fidler and Kripke 1977; Miller *et al.* 1983). More recently, a study of human breast tumours demonstrated that a single tumour contained heterogeneous subpopulations, in light of changes in DNA copy number. Among these subpopulations, few clones were identified to be associated with tumour expansion whereas most subpopulations of cells with variable pseudodiploid chromosome(s) were irrelavant to metastasis (Navin *et al.* 2011). Due to heterogeneity being common in single tumours, it is proposed that the origin of tumour heterogeneity might be analogous to that of organs and tissue, in which cells are heterogenous but arranged in a hierarchical structure. It has been well-established that a normal organ/tissue is derived from a small number of stem cells (SCs). Therefore, it is proposed that a bulk of tumour mass might be derived from

few tumour-initiating cells that inherit or acquire SC properties, including self-renewal, differentiation potential, and resistance to chemotherapy and radiotherapy (Reya *et al.* 2001).

Since the novel Cancer Stem Cell (CSC) theory is based on activities of SCs, the understanding of SC biology is necessary. The SCs can self renew through symmetric division to two SCs, thus maintaining SC pool and life span of SCs. To construct a whole organ or tissue, the SCs can undergo asymmetric division to simultaneously produce one SC and one differentiated progeny cells. Alternatively, SCs undergo committed symmetric division to produce to two differentiated progeny cells (Tang 2012). Based on differentiation potential, SCs are classified into totipotent, pluripotent, multipotent, oligopotent, and unipotent SCs. The differentiation potential of SC is correlated with cellular differentiation degree. The more primative a SC is, the more potent differentiation potential it has. In embryo, the cells at the zygote and the morula stages are totipotent. These totipotent SCs are able to differentiate to all embryonic and adult cells, thus constructing an entire animal body. As the morula cells undergo cell division, the embryo develops to form blastocyst. In blastocyst, the cells located at the inner cell mass are pluripotent and have the capability of producing cells of three germ layers, endoderm, mesoderm, and ectoderm. After the embryonic cells are partitioned into the three germ layers, most of these cells such as adult SCs become multipotent and have the capability of differentiation into cells of multiple but closely related lineages (Wagers and Weissman 2004; Tsonis 2007). However, there are few exceptions of certain adult SCs which can differentiate to cells crossing different germ layers. For instance, mesenchymal SCs of mesodermal origin, which give rise to not only mesenchymal cell lineages incuding osteocytes chondrocytes and adipocytes but also several cell lineages of three germ layers (Jiang *et al.* 2002; Wagers and Weissman 2004). The multipotent SCs can further differentiate into more committed progenitors with oligopotency. These oligopotent progenitors, such as common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) residing in bone marrow, have the capacity of producing few cell types. For instance, CLP can futher differentiate into

committed progenitors of B and T lymphocytes and natural killer cells. The unipotent progenitors are the descents of oligopotent progenitors and have the limited ability of differentiation potency, which is producing terminally differentiated cells. For instance, a B cell progenitor can differentiate into a B lymphocyte (Bryder *et al.* 2006).

Beyond the self-renewal and differentiation potential, SCs have been reported to be more resistant to chemotherapy and radiotherapy, due to the following reasons. In the first place, chemotherapeutic agents and radiation are used to damage DNA synthesis and preferrentially target dividing cells (Sausville and Longo 2001). However, SCs are slow-cycling and frequently at quiescent state (Cotsarelis *et al.* 1989). Secondly, a subset of SCs express several ATP-binding cassette (ABC) transporters that acts to extrude a variety of chemical compounds such as Hoechst dye 33342 and chemotherapeutic agents from cells, which is the leading cause of multi-drug resistance in SCs (Bunting 2002). Thirdly, SCs have been found to contain lower concentrations of ROSs than their non-SC counterparts. The lower ROS levels are correlated with higher free radical scavenge levels, resulting in cells being less sensitive to ionizing radiation-induced DNA damage (Diehn *et al.* 2009).

The CSC theory states that Cancer is derived from SCs with accumulation of molecular alterations such as genetic mutations and epigenetic changes. In other words, Cancer is a Stem Cell disorder. Like normal SCs, it is hypothesized that CSCs are able to self-renew and differentiate in an uncontrolled manner, which results in producing heterogeneous cancerous cells and thereby building a whole but abnormal organ/tissue. In contrast to CSCs, their more mature progeny cells which lose the ability to self renew and have restricted differentiation potential are less likely to produce cancer (Figure 1.11) (Polyak and Hahn 2006). Since SCs are chemoresistant and radioresistant due to slowly cycling, containing multi-drug resistance mechanism and lower ROS levels, it is presumed that CSCs inherit these properties, which may account for cancer recurrence after chemotherapy and radiation therapy (Sausville and Longo 2001; Bunting 2002; Diehn *et al.* 2009). In addition, accumulating evidence suggests that CSCs acquire radioresistance through molecular alterations, such as mutation of *p53* which is a DNA

damage checkpoint gene (Gorgoulis *et al.* 2005). For instance, the putative CSCs derived from human glioma xenograft and patients' specimens were found to be more resistant to ionizing radiation than their non-CSC counterparts, in that the CSCs acquired radition-induced DNA damage response mechanism that accelerated DNA repair whereas those non-CSCs that lacked this mechanism were radiosensitive (Bao *et al.* 2006).

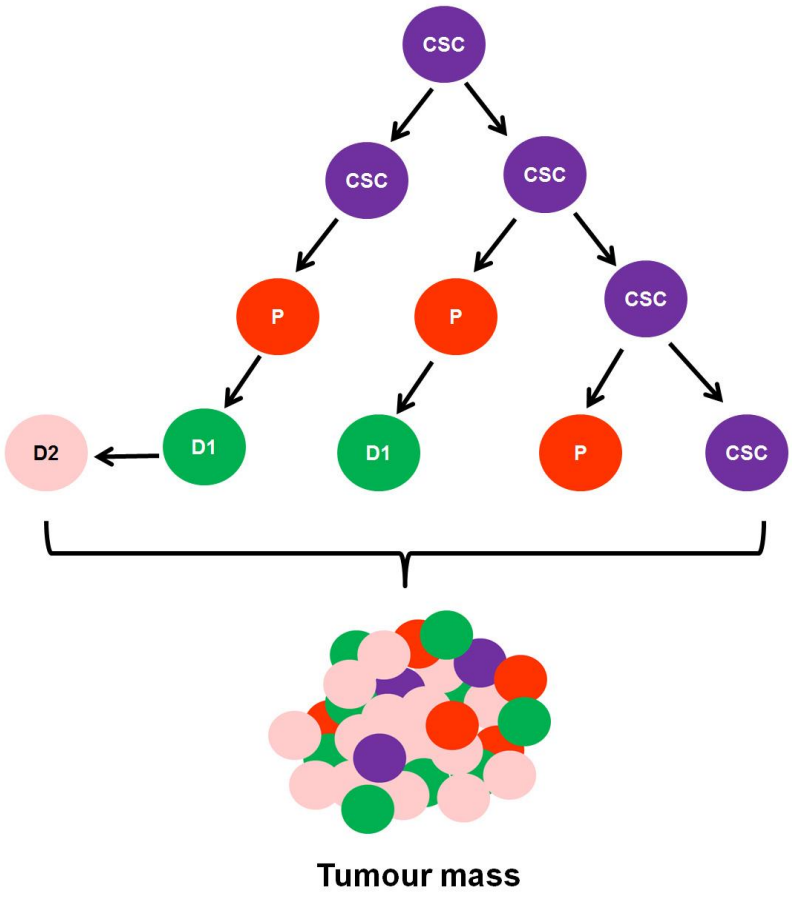


Figure 1.11 The model of cancer stem cell (CSC) theory. A small population of tumour cells with stem cell-like properties that can undergo both symmetrical and asymmetrical division, resulting in tumour cell heterogeneity. CSC, cancer stem cell; P, lineage committed progenitors; D1, incompletely differentiated tumour cells; D2, fully differentiated tumour cells.

1.8.2 CSC niche

In human and mouse, SCs have been found to reside in a specific microenvironment, termed SC niche, where serves as a reservoir of SCs by regulating self-renewal, proliferation and differentiation of SCs (Gould 2007; Borovski *et al.* 2011; Nagasawa *et al.* 2011). SC niche consists of various stromal cells such as fibroblasts and inflammatory cells, vascular-associated cells, soluble molecules, and extracellular matrix (Borovski *et al.* 2011). For example, adult neural SCs (NSCs) and progenitors reside in perivascular regions in the the hippocampus and the olfactory bulb, where neurogenesis takes place (Gould 2007). An *in vitro* study showed that co-culture of murine NSCs and murine endothelial cells promoted expansion of NSC colony. The phenomenon of self-renewal and colony expansion was contributed by endothelial cells that released endothelial factors and subsequently activated Notch/Hes1 signaling pathway in NSCs (Shen *et al.* 2004). Hematopoietic SCs (HSCs) are found to reside in the endosteal niche composed of bone-lining osteoblasts and the vascular niche composed of endothelial cells, reticular cells and nestin cells. It is suggested that these stromal and vascular cells may contribute to maintenance of undifferentiated state and the homing of HSCs (Nagasawa *et al.* 2011).

Like SC niche, recent studies suggest that CSC niche is presumed to exist and may execute several functions, including maintenance of CSC pool, up-regulation of CSC phenotype and contribution to metastasis (Borovski *et al.* 2011). For instance, the maintainence of the pool and self-renewal of CSCs derived from glioblastoma multiforme (GBM), which is characterized by necrotic foci surrounded by anaplatic astrocytic cells and vascular endothelial proliferation, has been suggested to be partly contributed by nitric oxide (NO) that is produced by intratumoural endothelial cells (Charles *et al.* 2010; Westphal and Lamszus 2011). It has been demonstrated that elevated production of NO activate Notch signaling cascades through NO/cGMP/PKC pathway in glioma cells and down-regulation of NO activity decrease Notch signaling (Charles *et al.* 2010). Moreover, an *in vitro* study of primary GBM specimens cultured

in three-dimensional explant system suggested that tumour endothelial cells were associated with promoting self-renewal and proliferation of CSCs by up-regulating Notch signaling (Hovinga *et al.* 2010). Although studies have shown that anti-angiogenesis therapy sensitizes glioma CSC to chemotherapy *in vivo* and amplifies cytotoxic effects of chemotherapy and prolongs survival time in phase II clinical trial of recurrent GBM (Folkins *et al.* 2007; Buie and Valgus 2008), a more recent study of mouse model of GBM and pancreatic neuroendocrine carcinoma has warned of risk to increase invasive and metastatic phenotypes in tumours after anti-angiogenesis therapy and suggested that hypoxia may be a key factor for tumour cells to escape anti-angiogenesis therapy (Paez-Ribes *et al.* 2009). In addition to vascular niche, it is proposed that CSCs may reside in the periphery of necrotic foci where hypoxic in GBM, based on the observation of preferential expression of *HIF2α* and other hypoxia response genes in CSCs derived from surgical gliomblastoma specimens and human tumour xenografts compared to non-CSC counterparts and physiological neural progenitors (Li *et al.* 2009; Borovski *et al.* 2011).

Recently, evidence has shown that microenvironement plays a key role in reprogramming differentiated tumour cells to CSCs and affecting cancer cell plasticity (Borovski *et al.* 2011). A study of colon cancer demonstrated that myofibroblasts secret hepatocyte growth factor and other unknown factors to maintain active Wnt signling in the neighbor CSCs and other differentiated tumour cells, thus maintaining CSC pool and driving differentiated tumour cells to express CSC phenotype (Vermeulen *et al.* 2010). Intratumoural hypoxia, as a microenrivonment factor, has been reported to not only encourage differentiated tumour cells to gain CSC phenotype but also enhance plasticity in cancer cell migration through induction of epithelial-mesenchymal transition (Heddleston *et al.* 2009; Taddei *et al.* 2013).

Increasing evidence suggests that foreign niches may express certain factors, which attract cancer cells to metastasize from the primary tumour site and to form new colonies. Some chemokines and their receptors have been found to be involved in cancer cell metastasis (Mishra *et al.* 2011). For instance, stromal cell-derived factor-1 (SDF-1),

a chemokine secreted by bone-lining osteoblasts and endothelial cells in bone marrow and by epithelial cells in organs, can be one of many mechasnisms for prostate cancer metastasis to bone, due to SDF1 attracting and transducing signal to its receptor CXCR4 which is expressed on the surface of prostate cancer cells (Nagasawa *et al.* 1998; Ponomaryov *et al.* 2000; Taichman *et al.* 2002).

1.8.3 Isolation of CSCs by sphere formation assay

Over the past fifteen years, putative CSCs have been identified within human leukaemia and solid tumours, and many cancer cell lines. Experimental methods, including sphere formation assay, colony formation assay, side population (SP) analysis, CSC maker, and xenograft assay, have been used to identify those tumour-initiating cells (Singh *et al.* 2003; Kondo *et al.* 2004; Collins *et al.* 2005; Fang *et al.* 2005; Seigel *et al.* 2005; Ho *et al.* 2007; Li *et al.* 2007).

1.8.3.1 Sphere formation assay

The sphere formation assay has been established to isolate and grow neural stem cells. This assay uses a non-adherent and serum-deprived culture system specifically developed to maintain SCs in an undifferentiated state. Additional constituents and growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) which are required for the expansion and self-renewal of neural SCs are added to the serum-deprived culture medium. The neural SCs proliferated under such culture condition look like spheres or a cluster of cells. Therefore, the neural SCs are also termed neural spheres. These neural spheres were demonstrated to be capable of self-renewal by being passaged to produce multiple new spheres after the old spheres were dissociated to single cells. These neural spheres were shown to express nestin which is a marker for neural SCs, but not the differentiated neural cell markers, including neuron-specific enolase (NSE), neurofilament (168 kD) and glial fibrillary acidic protein (GFAP). After the sphere cells were transferred to cell plates coated with an adhesion substrate poly-L-ornithine, these spheres were demonstrated as being multipotent and

differentiating into mature cell types including neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss 1992). To date, the sphere formation assay have been employed to isolate putative CSCs from many solid tumours, such as neurospheres isolated from brain tumours, sarcospheres from bone sarcomas and mammospheres from breast cancer (Singh *et al.* 2003; Gibbs *et al.* 2005; Grimshaw *et al.* 2008). Like neural spheres derived from mouse central nervous system, spheres derived from solid cancer were demonstrated as being capable of self-renewal and possessing differentiation potential. For instance, a clinical study showed that mammospheres derived from most (20/27) pleural effusion specimens of patients with metastatic human breast cancer could be passaged serially by using sphere formation assay. After these mammosphere were transferred to adherent plates, the sphere cells were differentiated to more mature cell types such as luminal epithelial, basal and myoepithelial cells. The mammospheres were demonstrated as being more potent to produce tumours than their differentiated progeny cells in severe combined immunodeficiency disease (SCID) mouse model (Grimshaw *et al.* 2008).

The published recipes for growing CSCs from brain, bone and breast cancers were quite similar. The basic serum-free medium utilized for isolating CSCs was N2 medium with addition of EGF. The N2 medium is composed of a 1:1 mixture of Ham's F12 medium and the Dulbecco's modified Eagle's medium (DMEM) supplemented with insulin, transferrin, progesterone, putrescine, and selenium (as Na_2SeO_3) (Bottenstein and Sato 1979; Reynolds and Weiss 1992; Singh *et al.* 2003; Gibbs *et al.* 2005; Chen *et al.* 2008; Grimshaw *et al.* 2008). EGF was demonstrated to be critical for expanding neural stem cell (NSC) population whereas medium plates coated with adhesive substrates such as poly-L-ornithine hindered proliferation of NSCs (Reynolds and Weiss 1992). Recently, a commercial available supplement B27/NS21 (for 21 different ingredients) has been widely used in serum-free culture system for growing breast and brain CSCs. The B27/NS21 contains not only all of the supplements in N2 medium and but also other ingredients to support neural cell growth (Chen *et al.* 2008). Other ingredients such as bFGF, sodium bicarbonate (as $NaHCO_3$), Hepes buffer were

documented in recipes of neurosphere and sarcosphere whereas hydrocortisone was added in mammosphere recipe (Singh *et al.* 2003; Gibbs *et al.* 2005; Grimshaw *et al.* 2008).

1.8.3.2 Colony formation assay

The *in vitro* colony formation assay, which is defined as semi-solid culture medium, has been employed to isolate hematopoietic and neural SCs (Ploemacher *et al.* 1989; Louis *et al.* 2008). This assay keeps all the advantages of the sphere formation assay, including maintainence of SCs in undifferentiated state, propagation of SCs and revelation of self-renewal capacity of SCs. Additional advantage of the colony formation assay is that it is capable of quantifying the actual frequency of SCs, in that SCs can stay in this culture system long enough (~ 21 days) to expand colony size. In contrast, the the maximal length of time for SCs to proliferate in sphere formation assay is 5-7 days, due to the development of central necrosis in spheres and disseminated cell debris after the spheres grow larger. Since the colony formation assay gives SCs adequate time to expand colony size, SCs are easily distinguished from their progeny cells such as progenitors and mature cells, based on self-renewal and proliferation capacities (Marshall *et al.* 2007; Louis *et al.* 2008).

1.8.3.3 Side population (SP) analysis

It is known that some tumour cells can survive after cancer therapy and contribute to the recurrence of tumours and metastatic diseases. One possible mechanism is that these tumour cells possess some drug-resistant mechanisms that can exclude drug from cells. The best characterized drug-resistance mechanism is that imparted by the superfamily of cell membrane-bound ABC transporters which are classified into seven subfamilies that are designated A to G. The subfamily B (ABCB), C (ABCC), and G (ABCG) of the ABC transporter superfamily confer a multidrug resistant mechanism to tumour cells (Vasiliou *et al.* 2009; Lebedeva *et al.* 2011). Interestingly, two ABC transporters, ABCB1 (also called MDR1) and ABCC1 (also

called BCRP), are commonly expressed by SCs and progenitors derived from bone marrow and skeletal muscle (Zhou *et al.* 2001; Bunting 2002). Since the ABCB, ABCC and ABCG subfamilies of ABC transpoters function in efflux of certain cytotoxic drugs and fluorescent dyes, the SP discrimination assay is employed to distinguish SCs or progenitors from their differentiated progeny cells which do not express ABC transpoters. The SP analysis identifies SCs and progenitors by comparison of fluorescent substrate (flow cytometry) profile of cells in the presence of ABCB1 and ABCC1 inhibitors with that of cells in the absence of these inhibitors. The SP cells are present in the fluorescent substrate profile when cells are treated with the inhibitors whereas no SP cells are found in the absence of the inhibitors. For instance, Hoechst33342 dye, as a substrate of both ABCB1 and ABCC1 transporters, was rarely accumulated in murine hematopoietic stem cells, due to efflux of this dye by both transporters. With addition of ABCB1 and ABCC1 inhibitors, these primitive cells could not pump out Hoest 33342 dye and restore the staining of these precursors, resulting in the presence of SP tail in Horchst33342 dye profile (Goodell *et al.* 1996).

To date, SP cells have been identified in many tissues and organs, including bone marrow (where SP cells comprise 0.1% of total bone marrow cells), skeletal muscle (2.3%), kidney (0.03~0.1%), umbilical cord blood (~0.4%), liver (~2% of non-parenchymal cells), mammary gland (~0.4%), lung (0.03~0.07%) and forebrain (3.6%) (Goodell *et al.* 1996; Storms *et al.* 2000; Asakura *et al.* 2002; Kim and Morshead 2003; Shimano *et al.* 2003; Summer *et al.* 2003; Clayton *et al.* 2004; Iwatani *et al.* 2004). Cumulative evidence suggests that SP cells are multipotent and have plasticity. For instance, a report showed that SP cells derived from murine skeletal muscle were able to differentiate into hematopoietic lineage cells after these SP cells were transplanted to bone marrow of mouse recipient (Jackson *et al.* 1999). SP cells derived from mouse bone marrow were able to differentiate to cardiomyocytes and endothelial cells and subsequently reconstitute functional cardiac tissue in a mouse model of myocardial infarction following transplantation (Jackson *et al.* 2001).

Despite the enrichment of cells with SC characteristics in the SP of some tissues, there are some conflicting data that do not support that SP cells represent SCs. Firstly, the data obtained from human and murine embryonic stem (ES) cells and epidermis suggested that SP cells might either be distinct from SCs or represent only a subgroup of SC pool. It was reported that human and mouse epidermal SP cells display phenotypes which were different from those of epidermal SCs (Terunuma *et al.* 2003; Triel *et al.* 2004). Moreover, whether SP phenomenon can serve as a SC marker continues to be questioned because ABCB1 transporter is normally expressed on the surface of certain differentiated epithelial and endothelial cells in tissue or organs, including small intestine, liver, kidney, placenta, endothelial cells in the blood-brain and blood-testes barriers (Cordon-Cardo *et al.* 1989; Kamimoto *et al.* 1989; Hsing *et al.* 1992; Okamura *et al.* 1993; Lankas *et al.* 1998; Choo *et al.* 2000). It was supposed that these cells might utilize ABCB1 transporter to pump out toxins and drugs, serving as a cell protection mechanism (Fromm 2000).

Recently, SP discrimination assay has been applied to CSC biology, in that tumours resistant to conventional therapy may be attributed to these CSCs displaying SP phenotype (Hirschmann-Jax *et al.* 2004; Chua *et al.* 2008). For instance, SP cells were identified in most neuroblastoma specimens (65%) from patient. These SP cells were capable of generating SP and non-SP cell populations *ex vivo*, excluded the chemotherapeutic drug Mitoxantrone and expressed higher levels of mRNAs encoding ABCA3 and ABCG2 transporters, as compared with non-SP cells (Hirschmann-Jax *et al.* 2004). Another example is that human astrocytoma SP cells expressed SC markers including nestin and musashi-1 and outcompeted for reconstitution of new tumours and aggressiveness. Interestingly, SP fraction was increased in response to chemotherapeutic agent Temozolomide, suggesting that the SP represents cells resistant to, and thus potentially selected by, conventional chemotherapy (Chua *et al.* 2008). In addition to brain cancers, SP cells were identified from a variety of tumour types, incluing breast, gastrointestinal, liver, lung, prostate, ovarian and thyroid cancers, representing 0.02-2.2% fraction of a whole tumour. These SP tumour cells were demonstrated to be more

competitive for recapitulation of new tumours *in vivo* as compared with their non-SP counterparts (Patrawala *et al.* 2005; Chiba *et al.* 2006; Haraguchi *et al.* 2006; Ho *et al.* 2007; Mitsutake *et al.* 2007; Bleau *et al.* 2009). For instance, 1000 – 10,000 SP cells identified from glioma and breast cancer recapitulated new tumours whereas 50,000-250,000 non-SP cells were required for tumour formation. It should be noted that not every tumour contain SP cells. In this study, SP cells were identified in ~30% of tumours (Patrawala *et al.* 2005).

1.8.3.4 CSC marker

Currently, many markers, such as nestin, CD34 and CD133, are used for identification of SCs and progenitors (Reynolds and Weiss 1992; Simmons *et al.* 1992; Lee *et al.* 2005). For example, CD133 is a marker used to identify SCs and progenitors derived from many organs and tissue, including bone marrow, brain, liver, muscle, prostate, kidney and cord blood (Mizrak *et al.* 2008). A study showed that CD133+ cells isolated from murine brain were able to form neurospheres and differentiated into multiple neural cells including astrocytes, oligodendrocytes and neurons, demonstrating self-renewal and multipotency of CD133+ murine neural cells (Lee *et al.* 2005). These markers for labeling normal primative cells are also used to identifiy putative tumour-initiating cells. Characterization of the phenotypes of a certain tumour-initiating cell is usually performed after the isolation of the spheres from SC culture and/or SP analysis. For instance, neurospheres derived from specimens of human brain cancers were demonstrated to express nestin and CD133 whereas their progeny cells expressed differentiated cell markers such as GFAP but lost expression of nestin and CD133 (Singh *et al.* 2003). Alternatively, tumour-initiating cells can be detected and isolated by directly staining with antibodies for markers which are known to identify normal stem cells. CD133+ hepatocarcinoma cells have been identified and considered to be tumour-initiating cells based on the previous demonstration of expression of this marker on normal liver stem cells. In the hepatocarcinoma stem cell study, the cell lines were not screened in advance by stem cell culture and SP analysis. Instead, they were staining

with CD133 surface marker and compared the ability of reconstitution of tumour types of CD133+ cells compared with CD133- cells *in vitro and in vivo* (Ma *et al.* 2007). In addition to CD133 and nestin, other makers for labeling tumour-initiating cells from various tumours have been identified. For examples, CSCs of AML are defined as co-expression of CD34+CD38-Thy-1 (Blair *et al.* 1997; Blair *et al.* 1998; Blair and Sutherland 2000; Jordan *et al.* 2000). The CSCs of human head and neck squamous cell carcinomas (HNSCC) carcinoma have phenotype of CD29+CD44+CD133+ (Harper *et al.* 2007).

1.8.3.5 Xenograft assay

In vivo xenograft assay is the gold standard assay to validate the CSCs identified by any of the four *in vitro* methods as described above (sphere formation assay, colony formation assay, SP analysis and labeling of SC marker). In order to prove whether CSCs possess SC properties, the xenograft assay is employed by initially fractionation of tumour cells into CSC and non-CSC populations. Subsequently, the two cell populations are serially diluted to various cell densities. After that, the two cell populations are injected into immunodeficient mice to evaluate tumour initiation potential. It is hypothesized that the CSC population with self-renewal, proliferation and differention properties are more capable of producing xenograft tumours which resemble the parent tumour than the non-CSC population. In addition, it is supposed that the xenograft tumours contain the CSC fraction which can then go on to form secondary xenografts successfully and the non-CSC fraction is expected to lose this tumourigeneicity (O'Brien *et al.* 2010). Increasing evidence supports the CSCs isolated by *in vitro* experimental methods are able to produce xenograft tumours in animal models. For instance, as few as (5000) single cells dissociated from the mammaospheres derived from pleural effusion specimens of human breast tumours were found to produce xenograft tumours in SCID mice. In contrast, and supporting the CSC hypothesis, the same number of the pleural effusion tumour cells did not produce tumours in SCID mice (Grimshaw *et al.* 2008).

1.8.4 Signaling pathways that regulate SCs and CSCs

1.8.4.1 Notch-Shh-TGFβ-Wnt-FGF signaling network

To date, several signaling pathways, including neurogenic locus notch homologue protein (Notch), sonic hedgehog (Shh), Wnt and class I PI3K pathways, have been reported to be relevant to regulation of normal SCs and CSCs. Stemness property of normal stem cells was regulated by a balance of Notch-Shh-TGFβ-Wnt-fibroblast growth factor (FGF) signaling network (Katoh 2007).

In neural SC biology, Notch signaling pathway plays a key role in keeping SCs at quiescent state and maintaining SC pool through inhibition of differentiation and proliferation of SCs. This pathway is activated following the Notch receptors (Notch1, Notch2, Notch3, and Notch4) binding to their ligands, including Delta-like protein 1 (DLL1), DLL2, DLL3, Jagged 1 and Jagged 2. The binding of ligand to Notch receptors results in the release of the signal-transducing Notch intracellular domain (NICD) from Notch receptor, allowing NICD to enter nucleus and subsequently interact with RBP-Jkappa family, thereby activating expression of genes encoding transcription factors Hairy and Enhancer of Split (HES) and Hairy/Enhancer-of-split-related with YRPW-like Motif (HEY). The HES and HEY family transcription factors acted as negative regulators of differentiation (Iso *et al.* 2003; Westphal and Lamszus 2011). However, emerging evidence suggested that Notch signaling might have a role in induction of neuronal differentiation (Furukawa *et al.* 2000; Morrison *et al.* 2000). For instance, multipotent neural crest stem cells were observed to undergo committed glial differentiation in response to DLL1 stimulation (Morrison *et al.* 2000).

Activation of Shh signaling is required for SCs to maintain self-renewal. Recent evidence suggested that active Shh signaling contributed to the growth of CSCs and tumour metastasis and invasion through regulation of EMT (Lee *et al.* 2008; Tang *et al.* 2012). The binding of Shh ligand to its receptor, Patched 1 (Ptch1), relieved inhibition of intrinsic Ptch1-mediated inhibition of Smoothened (Smo) receptor on cell surface. This

resulted in the dissociation of Gli family transcription factors from an inhibitory protein complex, allowing Gli to enter the nucleus to activate transcription of its target genes such as *Gli1* and *Ptch1* (Huse and Holland 2010; Stanton and Peng 2010).

TGF-β/bone morphogenetic protein (BMP) played a role in proliferation and differentiation during embryogenesis and was reported to induce EMT in cancer. The binding of TGF-β ligand to its receptor resulted in phosphorylation of SMAD family transcriptors. This promoted migration of SMAD to nucleaus, thus activation of expression of its target genes (Javelaud *et al.* 2011).

Wnt signaling has been reported to function in embryogenesis, tumourigenesis and tissue regeneration (Katoh 2007). Wnt signaling can be directed to canonical Wnt pathway to dictate cell fate or be directed to noncanonical Wnt pathway for cytoskeletal rearrangement and cell polarity. Canonical Wnt pathway is initiated by Wnt ligand-binding to its frizzled family receptors and LRP5/LRP6 coreceptor, which leads to the dissociation of its substrate β‑catenin from an inhibitory cytosolic complex composed of adenomatous polyposis coli (APC), Axin and GSK3β. This allows β‑catenin to migrate to nucleus and activate expression of its target genes such as *MYC, CCND1* (encoding cyclin D1), *FGF20* (Katoh 2007; Huse and Holland 2010).

The binding of FGF to its receptor FGFR, a subfamily of RTK, triggered activation of FGFR-mediated downstream pathways, including PLC-γ/PKC, class I PI3K/Akt, Ras/Raf, STAT and RSK2 pathways. As a result, FGF signaling modulated physiological cell functions such as proliferation, differentiation, mobility, survival and cell shape (Wesche *et al.* 2011).

1.8.4.2 The role of class I PI3K/Akt/mTOR pathway in regulation of SCs and CSCs

More recently, many reports have addressed that class I PI3K/Akt/mTOR pathway is involved in regulation of viability, proliferation, and multidrug resistance mechanism of CSCs (Bleau *et al.* 2009; Dubrovska *et al.* 2009; Martelli *et al.* 2010). A study of prostate cancer showed that spheres expressed higher phosphorylation levels of class I PI3K downstream components than adherent cells. Down-regulation of either PTEN or FoxO3a expression by short hairpin RNA (shRNA) increased in sphere production and up-regulated tumourigenic capability (Dubrovska *et al.* 2009). Akt, instead of mTOR, was found to up-regulate ABCG2 transporter activity, resulting in an increase in SP fractions in mouse and human gliomas (Bleau *et al.* 2009). Knockdown of PTEN in hematopoietic SCs resulted in leukemia-initating cell transformation. This disorder was mainly attributed to mTOR dysregulation, as evidenced by Rapamyin restoring functions of PTEN-deficient hematopoietic SCs (Yilmaz *et al.* 2006). Down-regulation or loss of PTEN expression resulting in production of tumour cells with stem cell-like properties has been observed in many cancer types, including brain, breast, liver, lung and prostate cancer and leukemia (Yilmaz *et al.* 2006; Zhang *et al.* 2006; Zhou *et al.* 2007; Yang *et al.* 2008; Bleau *et al.* 2009; Dubrovska *et al.* 2009; Korsten *et al.* 2009; Rountree *et al.* 2009).

1.8.5 Therapy targeting CSCs

As CSCs are assumed to be responsible for tumour relapse, development of strategies to effectively eliminate CSCs is required. These strategies include targeting CSC biomarkers, targeting CSC signaling pathways, sensitizing CSCs to therapy and targeting specific CSC metabolism (Baccelli and Trumpp 2012). Strategy to target CSC biomarkers, such as anti-CD44 antibody and anti-CD123 antibody, has been reported to effectively inhibit relapse of xenograft of human acute myeloid leukemia (AML) in mouse model (Jin *et al.* 2006; Jin *et al.* 2009). However, due to CD44 and CD123 markers being expressed not only on CSCs but also on normal SCs, it is speculated that

these antibodies may affect normal SCs and have adverse reactions on patients (Taussig *et al.* 2005; Orian-Rousseau 2010).

Based on the theory that CSCs may rely heavily on certain signaling pathways to maintain stemness and self-renewal as compared with their normal counterparts, strategy to target these pathways is under investigation (Weinstein 2002; Baccelli and Trumpp 2012). For instance, previous studies of brain tumours showed that suppresion of Notch pathway by γ-secretase inhibitors remarkably reduced or complete depleted the CSC fraction in *in vitro* experiments and significantly inhibited the growth of tumour xenografts of brain CSCs in mouse model (Fan *et al.* 2006; Fan *et al.* 2010). Promoting CSCs to undergo differentiation is an alternative way to reduce the CSC fraction. For instance, activation of bone morphogenetic protein (BMP)/Smad pathway by BMP4 promoted differentiation, decreased proliferation but not affected viability, of CSCs derived from human GBM. Furthermore, intracranial delivery of BMP4 effectively inhibited the growth of GBM xenografts in mouse model (Piccirillo *et al.* 2006).

Strategy to sensitize CSCs to radiotherapy/chemotherapy is currentlty being assessed in preclinical studies. For instance, inhibition of Notch signaling by γ-secretase inhibitor-I has been observed to augment radiosensitivity of radioresistant CSCs isolated from glioblastomas. Moreover, pretreatment of unsorted glioblastoma cells with Notch inhibitor and radiation remarkably reduce tumor incidence in mouse model, as compared with single treatment groups and control cells (Lin *et al.* 2010). Recently, promyelocytic leukaemia protein (PML) tumour suppressor has been found to be crucial for maintaining stemness of HSCs and leukaemia SCs (LSCs. *In vitro* and *in vivo* results revealed that pretreament of LSCs with arsenic trioxide (As_2O_3), resulting in decreasing expression of PML and initiates differentiation of LSCs, sensitized LSCs to chemotherapy and delayed tumour incidence (Ito *et al.* 2008).

Strategy to target specific CSC metabolism such as nitric oxide synthase-2 (NOS2) in glioma SCs and glycine decarboxylase in CSCs derived from non-small cell lung cancer, is being investiageted in preclinical studies (Eyler *et al.* 2011; Zhang *et al.*

2012). For instance, evidence has shown that nitric oxide (NO), produced by intratumoural endothelial cells through endogenous NOS3 enzyme, maintains stemness of CSCs derived from malignant glioma (Charles *et al.* 2010; Eyler *et al.* 2011; Westphal and Lamszus 2011). In addition, higher expression levels of NOS2 enzyme, which acts to produce NO, has been detected in glioma CSCs than their differentiated progeny cells. Furthermore, inhibition of NOS2 activity did arrest the growth and proliferation of glioma CSCs *in vitro* and reduced the tumourigenecity *in vivo* (Eyler *et al.* 2011).

1.8.6 Controversy of cancer stem cell theory

Although many studies support CSC theory based on the evidence showing SC-like tumour cells being more tumourigenic than their differentiated progeny cells (Bonnet and Dick 1997; Al-Hajj *et al.* 2003; Singh *et al.* 2003; Grimshaw *et al.* 2008), the findings of two recent studies challenge this CSC concept (Kelly *et al.* 2007; Yoo and Hatfield 2008). One study showed that as few as 10-100 randomly chosen tumour cells from murine lymphoma or leukaemia developed tumour in histocompatible mice whereas no tumour incidence was detected mice injected with control group (normal spleen cells) (Kelly *et al.* 2007). The other study showed that as few as 1-10 unsorted single cells from murine lung cancer or breast cancer form colonies *in vitro*. Subsequetly, 2 x 10^5 cells from each tumour colony were injected to histocompatible mice, which led to tumour formation (Yoo and Hatfield 2008). The results of these two studies suggest that tumourigenecity (the capability of generating tumours) may not necessarily be confined to a rare population of CSCs.

Increasing evidence suggests that CSCs are composed of heterogenous subgroups, instead of one single clone. Besides, it is suggested that CSC model is dynamic and tumour undergoes evolution during disease progression (Mullighan *et al.* 2008; Anderson *et al.* 2011; Notta *et al.* 2011). Recently, two studies of human leukaemia have discovered that genetic diversity exists in CSCs. Furthermore, it has been observed that a predominant CSC clone in an initial diagnostic tumour may remain

predominant clone or replaced by minor CSC subclone in recurrent tumour(s) after cancer therapy and tumour xenografts after serial transplantations in immunodeficient mouse model. Both studies have suggested that genetic diversity within CSC subclones allows tumour cells to develop drug resistantance mechanism, survive and adapt stringent milieu, and metastasize to distant sites (Anderson *et al.* 2011; Notta *et al.* 2011). Another example to support diverse heterogeneity within CSCs is the markers expressed by CSCs derived from human breast cancers. Previously, two studies employed different approaches to isolate CSCs from human breast cancer and gain two CSC phenotypes, aldehyde dehydrogenase 1 (ALDH1)-positive and $CD44^{high}CD24^{low}Lineage^{-}$, respectively. In one study, the population of CSC expressing $CD44^{high}CD24^{low}Lineage^{-}$ phenotype represented 11-35% of total tumour cell population (Al-Hajj *et al.* 2003). In the other study, 3-10% of CSCs expressing ALDH1 were detected in total tumour cells. Moreover, only 0.1-1.2 % of breast cancer cells exhibiting both CSC phenotypes of ALDH1 and $CD44^{high}CD24^{low}Lineage^{-}$, suggesting that most cells of the two CSC groups did not overlap. The results of *in vivo* experiment showed that CSCs expressing ALDH1 alone or both phenotypes were tumourigenic whereas ALDH-negative tumour cells expressing $CD44^{high}CD24^{low}Lineage^{-}$ did not initiate tumour (Ginestier *et al.* 2007). Although breast tumour cells expressing ALDH1 were generally more tumourigenic than those expressing $CD44^{high}CD24^{low}Lineage^{-}$, the cells expressing $CD44^{high}CD24^{low}$ were demonstrated to produce mammosphere in sphere formation assay and express EMT phenotype (Grimshaw *et al.* 2008; Mani *et al.* 2008).

The third issue about the controversy of CSC theory is that some extrinsic factors such as microenvironment and intrinsic factors genetic instability may have considerable impact on increasing the fraction of CSCs, leading to directing radiosensitive tumour cells to evolving to radioresistant cells, and enhacing invasive and metastatic phenotypes (Baccelli and Trumpp 2012). Indeed, it has been demonstrated that differentiated tumour cells can aquire CSC phenotypes through reprogramming process (Baccelli and Trumpp 2012). Induction of EMT, by either TGFβ1 treatment or ectopic expression of Snail or Twist transcription factors, allowed immortalized human mammary epithelial cells

(HMLEs) to gain the phenotype ($CD44^{high}CD24^{low}$) of mammary SCs and CSCs. In the same study, expression of EMT-associated genes, such as up-regulation of genes encoding vimentin, Snail, Slug, Twist and N-cadherin and down-regulation of E-cadherin, was detected in SCs isolated from normal human and mouse mammary tissues as well as CSCs isolated from human breast carcinomas (Mani *et al.* 2008). In addition, intratumoural microenvironment may release factors which can maintain or increase self-renewal and stemness of tumour cells. For instance, hepatocyte growth factor released by intratumoural myofibroblasts has been found to maintain active Wnt signaling in the neighbor CSCs and other differentiated tumour of colon cancer, thus maintaining CSC pool and driving differentiated tumour cells to express CSC phenotype (Vermeulen *et al.* 2010).

Taken together, as tumour relapse and metastasis remain to be the major problem to solve, it is suggested that tumour evolution during disease progression, existence of genetically diverse subclones in CSCs, genetic instability in tumour cells, and micronenvironment factors should be considered, in addition to CSC concept, for future development of new strategy to treat cancer.

1.9 Research hypothesis

It is evident that class I PI3K regulates a wide range of cellular processes, including cell cycle progression, survival, protein synthesis, motility, metabolism and autophagy, via its two major downstream effectors Akt and mTOR (Huang and Houghton 2003; Manning and Cantley 2007; Engelman 2009). In recent decades, overexpression of class I PI3K/Akt/mTOR pathway as a result of alterations of the components of this pathway has been implicated in tumourigenesis in various cancer types in human, mouse and canine species. More recently, emerging evidence suggests overexpression of class I PI3K pathway may positively maintain CSC viability and confer multidrug reasistance of CSCs (Bleau *et al.* 2009; Dubrovska *et al.* 2009). In preclinical studies, inhibition of class I PI3K/Akt/mTOR axis signaling by utilizing small molecular inhibitors or small interfering RNA (siRNA) has been demonstrated to significantly reduce cell viability *in vitro* or tumour regression *in vivo* (Bjornsti and Houghton 2004; Altomare and Testa 2005; Bertelsen *et al.* 2006). In addition, some of this pathway inhibitors, such as Temsirolimus (CCI-779) and Everolimus (RAD001), have been approved by FDA and the European Medicines Agency for the treatment of advanced renal cell carcinoma and advanced pancreatic neuroendocrine tumours (Yuan *et al.* 2009; Saif 2011; Yao *et al.* 2011).

Therefore, the hypothesis underlying this research is that canine tumourigenesis is class I PI3K/Akt/mTOR axis pathway-dependent. Blockade of this pathway by utilizing selective small molecule inhibitors can prevent proliferation and survival of canine tumours.

1.10 Research aims

- Investigation into the role of the class I PI3K/Akt/mTOR pathway in canine tumourigenecity *in vitro*
- Evaluation of the efficacy of class I PI3K pathway inhibitors against cell viability in canine tumours *in vitro*
- Investigation into the mechanism of class I PI3K pathway inhibitors
- Evaluation of the efficacy of the combined therapy of two class I PI3K pathway inhibitors for canine tumours *in vitro*
- Evaluation of the efficacy of the PI3K pathway inhibitors in combination with chemotherapy for the treatment of canine tumours *in vitro*
- Investigation into the activity of the class I PI3K/Akt/mTOR pathway in the neurosphere versus adherent cells derived from canine glioma cell line.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

2.1.1 Cell lines and growth medium

Jurkat T (human acute lymphoblastic leukemia of T cell orgin, 3132 (canine lymphoma of B-cell origin), REM134 (canine mammary carcinoma), SB (canine hemangiosarcoma), J3T (canine glioma) and C2 (canine mast cell tumour) cells, were used in this study (Table 2.1). The Jurkat T, 3132, REM134 and J3T cells were grown in RPMI1640 (11835, Gibco, Invitrogen, Paisley, UK), RPMI 1640 (11835, Gibco, Invitrogen, Paisley, UK), DMEM (41965, Gibco, Invitrogen, Paisley, UK) and DMEM (41966, Gibco, Invitrogen, Paisley, UK) media respectively, all of which contained 10% v/v (volume to volume) fetal bovine serum (FBS), 100 unit (U)/milliliter (ml) penicillin and 100 μg/ml streptomycin. The C2 cell line was grown in Minimum Essential Medium Eagle (M5650, Sigma-Aldrich, Ayrshire, UK) medium containing 5% FBS, 1% non-essential amino acid mix (NEAA) (Sigma-Aldrich, St Louis, MO, USA), 1% GlutaMAX-1 (Invitrogen, Paisley, UK), 50 microgram (μg)/ml gentamicin. The SB cell line was grown in EBM-2 (CC-3135, Lonza, Basel, Switzerland) supplemented with 2% FBS and EGM-2 SingleQuots (CC-4176, Lonza, Basel, Switzerland) kit containing 0.04% hydrocortisone, 0.4% hFGF, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000 and 0.1% heparin.

Table 2.1. Cell lines used in this study

Cell line	Origin	Source	Culture medium
Jurkat T	acute lymphoblastic leukemia of T cell orgin	(Schneider *et al.* 1977)	RPMI1640
3132	canine lymphoma of B-cell origin	(Strandstrom and Rimaila-Parnanen 1979)	RPMI1640
REM134	canine mammary carcinoma	(Else *et al.* 1982)	DMEM
J3T	canine glioma	(Berens *et al.* 1999; Rainov *et al.* 2000)	DMEM
SB	canine hemangiosarcoma	(Akhtar *et al.* 2004)	EBM-2/EGM-2
C2	canine mast cell tumour	(DeVinney and Gold 1990)	Minimum Essential Medium Eagle

2.1.2 Cells from liquid nitrogen

Eppendorfs containing cryopreserved cells were taken out of liquid nitrogen and stored in dry ice until ready for cell culture. The growth medium and sterilized 1 x phosphate buffered saline (PBS) were warmed in water bath to 37 ^{o}C. The eppendorfs were quickly thawed in water bath. 70% ethanol was sprayed on the eppendorfs and the bottles of growth media and PBS for disinfection before they were placed in a Class 2 Biological Safety Cabinet. After that, the defrosted cells were transferred from the eppendorffs to a 15 ml tube. 9 ml of PBS was pipetted into the 15 ml tube and mixed with the cells. Subsequently, the 15 ml tube was spun in a centrifuge at 1500 rpm for 5 mins at room temperature. The supernatant was discarded. The cell pellet was resuspended in 7 ml of growth medium, followed by transferred to a T25 flask and cultured at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

2.1.3 Passaging cells

Growth media and 0.25% Trypsin-EDTA (1x) containing phenol red (Cat. No. 25200, Gibco) were stored at 4 ^{o}C. Sterilized PBS was stored at room temperature. Before cells were passaged, all the reagents, including growth media, Trypsin-EDTA and sterilized PBS, were warmed in water bath to 37 ^{o}C. 70% ethanol was sprayed on the surface of all the reagents and equipments for disinfection before they were placed in a Class 2 Biological Safety Cabinet.

2.1.3.1 Passaging cells- Adherent cells

When adherent cells, such as REM134, J3T, and SB cells, were grown at 80-95% confluency in a flask, cells were passaged. Growth medium was taken off by pipetting and cells were washed twice with PBS. Subsequently, various volumes (T25 flask– 1 ml, T75 flask- 2 ml, T150 flask- 4 ml) of 0.25% Trypsin-EDTA were added to the flask, ensuring that the Trypsin-EDTA covered the monolayer of cells. Then, the flask was incubated at 37 ^{o}C with 5% CO_2 for 3-5 mins until cells were detached. An equal

volume of FBS-containing growth medium was mixed with 0.25% Trypsin-EDTA in the flask, in order to terminate trypsinization. Cell suspension was transferred to a 50 ml tube and spun in a centrifuge at 1500 rpm for 5 mins at room temperature. The supernatant was discarded and cell pellet was resuspended in 10 ml of growth medium. For splitting cells at the ratio of 1:10, 1 ml out of the 10 ml cell suspension was transferred from the 50 ml tube to a new flask which contained the appropriate volumes of fresh growth medium (T25 flask- 6 ml, T75 flask- 12 ml, T150 flask- 27 ml). Then, the flask was incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

2.1.3.2 Passaging cells- Non-adherent cells

When non-adherent (suspension) cells, including Jurkat T and 3132 cells, were grown to 80% confluency, the whole cell suspension was transferred from the flask to a 50 ml tube and spun at 1500 rpm for 5 mins at room temperature. The supernatant was discarded and cell pellet was resuspended in 10 ml of growth medium. For splitting cells at the ratio of 1:10, 1 ml out of the 10 ml cell suspension was transferred from the 50 ml tube to a new flask containing the appropriate volumes of fresh growth medium (T25 flask- 6 ml, T75 flask- 12 ml, T150 flask- 27 ml). Then, the flask was incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

2.1.3.3 Passaging cells- C2 cell line

60% of C2 cells were adherent whereas 40% of C2 cells were non-adherent. As for cell passage, the whole cell suspension was transferred the cell suspension from the flask to a 50 ml tube. Then, the flask was washed twice with PBS. Various volumes (T25 flask– 1 ml, T75 flask- 2 ml, T150 flask- 4 ml) of 0.25% Trypsin-EDTA were added to the flask. Then, the flask was incubated at 37 ^{o}C with 5% CO_2 for 3-5 mins until cells were detached. An equal volume of FBS-containing growth medium was mixed with 0.25% Trypsin-EDTA in the flask, in order to terminate trypsinization. The whole cell suspension was transferred from the flask to the 50 ml tube which contained non-adherent C2 cells, followed by spun in a centrifuge at 1500 rpm for 5 mins at room

temperature. The supernatant was discarded and cell pellet was resuspended in 10 ml of growth medium. For splitting cells at the ratio of 1:10, 1 ml out of the 10 ml cell suspension was transferred from the 50 ml tube to a new flask containing the approapriate volumes of fresh growth medium (T25 flask- 6 ml, T75 flask- 12 ml, T150 flask- 27 ml). Then, the flask was incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

2.1.4 Counting cells

Cells were counted by using Neubauer improved haemacytometer (bright-lined, with spring clips). For adherent cells and the C2 cell line, cells were counted following the termination of trypsinization (see Chapter 2, Section 2.1.3.1 and 2.1.3.3).

A cover slip was placed on top of both counting chambers of the haemacytometer. 15 µl of 0.4% Trypan Blue (Sigma-Aldrich) was mixed with 15 µl of cell suspension. 10 µl of the mixture was pipetted into each counting chamber. Cells were counted in the central 25 squares, which contained 0.1 µl of the mixture, in each counting chamber. Then, the following formula of cell count was used:

Total number of cells per ml = (Total number of cells in the central 25 squares in both chambers) x 10^4

2.1.5 Cryopreservation

After cells were counted, cells were spun at 1500 rpm for 5 mins at room temperature. The supernatant was discarded and the cell pellet was resuspended in growth medium at the density of 6 x 10^6 cells/ml. 0.5 ml of cell suspension which contained 3 x 10^6 cells was stored in 0.5 ml of freezing medium composed of 10% dimethyl sulfoxide (DMSO) and 90% FBS in an 1.5 ml vial. The vial containing cells and freezing medium was placed in a pre-cooled (4^{o}C) isopropanol-filled

cryopreservation container and stored at -70^{o}C for 1-2 days. Then, the vial was stored in liquid nitrogen.

2.2 Sphere cell culture

All work of Section 2.2 Sphere cell culture courtersy of Dr. Lisa Pang

2.2.1 Growing J3T sphere and growth medium

Before cells were passaged, all the reagents, including N2 growth media, 0.25% Trypsin-EDTA and sterilized PBS, were warmed in water bath to 37 ^{o}C. 70% ethanol was sprayed on the surface of all the reagents and equipments for disinfection before they were placed in a Class 2 Biological Safety Cabinet.

The pellet of canine J3T cells was resuspended in 2 ml of sterilized PBS following trypsinization was terminated and cells were spun in a centrifuge (see Chapter 2, Section 2.1.3.1). Then, the cell counting method was performed (see Chapter 2, Section 2.1.4). Cells were spun in a centrifuge at 1500 rpm for 5 mins at room temperature. The supernatant was discarded and the cell pellet was resuspended in serum-free N2 medium with 0.8% methylcellulose at the density of 3 x 10^5 cells/ml. For this, a 1:1 mixture of 1.6% methylcellulose (Sigma-Aldrich) and 2X DMEM/F12 medium (Sigma-Aldrich) supplemented with 20 nM progesterone (Sigma-Aldrich), 100 µM putrescine dihydrochloride (Sigma-Aldrich), 30 nM sodium selenite (Sigma-Aldrich), 25 µg/ml human apo-Transferrin (Sigma-Aldrich), 20 µg/ml insulin from bovine pancreas (Sigma-Aldrich), 2.4 mg/ml sodium bicarbonate (Sigma-Aldrich), 10 nanogram (ng)/ml human EGF (PeproTech, Rocky Hill, USA) and 10 ng/ml human bFGF (PeproTech, Rocky Hill, USA).

2 ml of cell suspension which contained 60,000 cells was pipetted into each well in six-well Ultra Low Attachment plates (Corning) and incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2. Sphere cells were fed with 10 µl of 10 ng/ml

human EGF and 10 ng/ml human bFGF in each well every 3 days. After culture for 1-2 weeks, colonies were counted under light microscope.

2.2.2 Passaging spheres

2 ml of PBS was added to each well and mixed with the growth medium, in order to reduce the viscosity of N2 grwoth medium. The cell suspension was collected and transferred to a 15 ml tube. Another 2 ml of PBS was added to the tube and the tube was spun at 1000 rpm for 5 mins at room temperature. Supernatant was discarded and the cell pellet was resuspended in 0.5 ml of 0.05% Trypsin-EDTA, followed by incubated at 37 ^{o}C for 10 mins or until spheres were dissociated into sigle cells. 0.5 ml of FBS-containing medium was added to the tube for termination of trypsinization. After that, sphere cells were counted (see Chapter 2, Section 2.1.4). Cells were spun in a centrifuge at 1000 rpm for 5 mins at room temperature. Supernatant was discarded and the cell pellet was resuspended in serum-free N2 medium with 0.8% methylcellulose at the density of 3 x 10^5 cells/ml. 2 ml of cell suspension which contained 60,000 cells was pipetted into each well in six-well Ultra Low Attachment plates and incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

2.3 Drug compounds and pathway inhibitors

ZSTK474 (pan-PI3K inhibitor, Z-1066, LC Laboratories, USA) and Wortmannin (pan-PI3K inhibitor, 1232, Tocris bioscience, USA), and KP372-1 (Akt inhibitor, B-0102, Echelon, USA), all of which were supplied in powder forms, were made up to 40 millimolar (mM) stock solutions in DMSO and stored in 15 μl aliquots at -70 ^{o}C. Rapamycin (mTOR inhibitor, R0395, Sigma-Aldrich, USA), which was supplied in powder form, was made up to 20 mM stock solution in DMSO and stored in 10 μl aliquots at -70 ^{o}C. All the stock solutions of the inhibitors, including ZSTK474, Wortmannin, KP372-1 and Rapamycin were diluted freshly in cell medium before use.

Doxorubicin at 2 mg/ml concentration was purchased from Pharmacia, Pfizer Service Company (Zaventem, Belgium) and was soluble in water.

2.4 Cell viability assay

Cells were seeded at a density of 3 x 10^3 cells per well in 96-well plates overnight at 37^{o}C with 5% CO_2, followed by incubated with various doses of either single agent or in combination with other drugs, or DMSO vehicle for a period of time. All experiments were performed in at least three replicates. After the drug treatment, the number of viable cells was determined by using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. This commercial kit quantified cell viability by measuring the amount of ATP released from viable cells. The more viable cells were present, the more ATP released and the higher the value of luminescence detected.

2.5 Analysis of apoptosis and cell death

Cells were plated at a density of 3 x 10^4 cells per ml and incubated overnight at 37^{o}C with 5% CO_2. After that, cells were exposed to 20 micromolar (µM) ZSTK474 for 2 days, 400 nanomolar (nM) KP372-1 for 1 day, 20 µM Rapamycin for 2 days or vehicle control and then were collected for apoptosis analysis by using FITC Annexin V Apoptosis Detection Kit I (556547, BD Pharmingen™, San Diego, CA, USA). In brief, harvested cells were washed with cold PBS and re-suspended in 100 µl of 1x Binding Buffer, followed by stained with FITC Annexin V antibody and propidium iodide (PI) for 15 min in the dark at room temperature, according to the manufacturer's instructions. Cells were analyzed by flow cytometry using FACS Calibur Flow Cytometer and CellQuest software (BD Biosciences, San Jose, California).

2.6 Western blotting

2.6.1 Cell lysis

Cells were seeded at a density of 20,000 cells per ml overnight at 37 ^{o}C with 5% CO_2, and then incubated with various doses of either single agents or combinations of other drugs, or DMSO vehicle for a period of time. After the drug treatment, the cells were prepared for cell lysis. For adherent cell lines, medium was discarded and cells were rinsed with 1 ml cold PBS. Then, the cold PBS was taken off and 1 ml fresh cold PBS was added to cells. After that, cells were harvested by using cell scrapers and transferred to an 1.5 ml eppendorf, which was placed on ice. For suspension cell lines, cells were spun down in Centrifuge for 5 mins at the rotational speed of 1500 rpm at 25 ^{o}C. Then, medium was discarded. Cells were resuspened in 1 ml cold PBS and transferred to 1.5 ml eppendorf, which was placed on ice.

Cells in cold PBS in 1.5 ml eppendorfs were centrifuged for 5 mins at the speed of 5000 rpm at 4 ^{o}C and the supernatant was removed. After that, cell pellets could be either lyzed by using Nonidet-P40 (NP40) lysis buffer, or snap frozen in dry ice for 5 mins and stored at -70^{o}C.

The 1% NP40 lysis buffer contained 1% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO), 150 millimolar (mM) KCl, 25 mM Hepes (pH 7.4), 5 mM dithiothrietol (DTT), and 50 mM NaF. This lysis buffer was stored in 450 µl aliquots at -20 ^{o}C. In addition, 10 x Complete Mini Protease Inhibitor Cocktail Tablet (Roche, Mannheim, Germany) was dissolved in 500 µl distilled water, dispensed into aliquots (50 µl) and stored at -20 ^{o}C. To lyse cells, one volume of 10 x Complete Mini Protease Inhibitor Cocktail Tablet and nine volumes of 1% NP40 lysis buffer were thawed and mixed well. Then, twice the pellet volume of 1% NP40 buffer which contained 1 x Complete Mini Protease Inhibitor Cocktail Tablet was added to the cell pellet. The cell pellet was mixed thoroughly by pipetting in the lysis buffer and the pipetting process should be performed on ice. Then, cell pellet in lysis buffer was incubated on ice for 15 mins and centrifuged at 14000 rpm for 15 mins at 4 ^{o}C. After that, the supernatant was aspirated and

transferred to 1 ml eppendorff, followed by snap frozen in dry ice. All frozen supernatant samples were stored at -70 ^{o}C.

2.6.2 Protein quantification

The protein extracts were quantified by using Quick Start Bradford Protein Assay (Biorad Laboratories, CA, USA) according to the manufacturer's instruction. In the first place, samples were prepared as 1/20 and 1/40 dilutions. For 1/40 dilution, 19.5 µl distilled water was mixed with 0.5 µl sample by pipetting. For 1/20 dilution, 19 µl distilled water was mixed with 1 µl sample by pipetting. Volumes prepared were sufficient for two replicates.

Secondly, 2 mg/ml bovine serum albumin (BSA) standards and serial dilutions were prepared as shown in Table 2.2, according to the manufacturer's instruction. Volumes prepared were sufficient for three replicates.

Table 2.2. BSA standards and serial dilutions

Tube	Standard volume (µl)	Source of Standard	Diluent volume (µl)	Final [protein] (µg/ml)
1	20	2 mg/ml stock	0	2000
2	30	2 mg/ml stock	10	1500
3	20	2 mg/ml stock	20	1000
4	20	Tube 2	20	750
5	20	Tube 3	20	500
6	20	Tube 5	20	250
7	20	Tube 6	20	125
8	0	none	20	0

Table 2.2 was reproduced from Quick Start™ Bradford Protein Assay Instruction Manual (Biorad Laboratories, CA, USA).

5 µl of diluted samples and standards were dispensed to a clear 96-well plate. Then, 250 µl of Quick Start™ Bradford Dye Reagent (BioRad) was mixed with samples and BSA standards. The 96-well plate was read on a plate reader by measuring the absorbance at 595 nanometer (nm). Protein concentration of each tested sample was determined from a standard curve, which was produced from plotting the mean absorbances for BSA standards.

2.6.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.3.1 Making resolving gels

Gel cassettes and glass plates were assembled for gel casting. After that, 6%, 10% and 15% resolving gels (10 ml) were prepared using the following recipes shown in

Table 2.3. As for the reagents for making resolving gels, 30% (w/v) acrylamide/bis-acrylamide (National Diagnostics, UK) was stored in a dark area at room temperature. 1.5M Tris-HCl (pH 8.8) and 10% (w/v) sodium dodecyl sulphate (SDS) (Sigma-Aldrich) were stored at room temperature. 10% (w/v) ammonium persulfate (APS) (Sigma-Aldrich) was stored at 4 ^{o}C. N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich) was protected from light and stored at 4 ^{o}C.

It should be noted that both of 10% APS and TEMED were added to the resolving gel solution right before the gel solution was poured between the assembled glass plates, because both reagents could cause gel polymerization. Then, isopropanol was overlaid on the gel solution which was poured between the assembled glass plates, in order to prevent air bubbles existing in the gel. The gel solution was allowed to polymerize for 30-45 mins. After that, isopropanol was discarded and stacking gel solution was prepared.

Table 2.3. Recipes for making 10 ml resolving gels.

	6% (ml)	10% (ml)	15% (ml)
Distilled water	5.3	4	2.35
30% (w/v) acrylamide/bis-acrylamide	2.0	3.3	4.95
1.5M Tris-HCl pH 8.8	2.5	2.5	2.5
10% (w/v) SDS	0.1	0.1	0.1
10% (w/v) APS	0.1	0.1	0.1
TEMED	0.008	0.004	0.004

The resolving gels were used to separate a variety of molecular weight proteins. Lower molecular weight proteins required higher percentage resolving gels, which contained smaller pore sizes, for resolution.

2.6.3.2 Stacking gels

5% stacking gel was prepared using the following recipe (Table 2.4). 10% APS and TEMED, which acted to initiate gel polymerization, were added to staking gel solution right before the gel solution was poured and overlaid on the resolving gel in the assembled glass plates. Then, a comb was placed between the assembled glass plates, for the purpose of creating wells for loading samples. The stacking gel solution was allowed to polymerize for 30 mins. After polymerization of the stacking gel was completed, the whole SDS gel set which was composed of staking and resolving gels was ready for electrophoresis. Alternatively, the whole SDS gel set with comb could be wrapped in cling film and stored at 4 ^{o}C for 1-2 days.

Table 2.4. Recipes for making a 5 ml stacking gel.

	5% (ml)
Distilled water	3.4
30% (w/v) acrylamide/bis-acrylamide	0.83
1 M Tris-HCl pH 6.8	0.63
10% (w/v) SDS	0.05
10% (w/v) APS	0.05
TEMED	0.005

2.6.3.3 Sample and Running buffer prepared for electrophoresis

16 ml of 4 x Sample Buffer which contained 4 ml of 20% SDS, 4 ml of 1 M Tris-HCl (pH 6.8), 5 ml of 80% glycerol, 2 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA), and 1 ml of 1% bromophenol blue. At each use, 800 µl of 4 x Sample Buffer

was mixed with 200 µl of fresh 1M DTT. Protein samples were thawed on ice. Then, 1 volume of 50 µg protein sample was mixed thoroughly with 1 volume of 4 x Sample Buffer that contained DTT. Pre-stained molecular weight markers and all of the samples with Sample Buffer were heated to 100 ^{o}C for 2-3 mins. In this study, two types of pre-stained molecular weight markers were used; the pre-stained, broad range SDS-PAGE standards (Cat No. 161-0318, BioRad) and the Amersham full-range rainbow molecular weight marker (Cat No. RPN800E, GE Healthcare). After all of the samples were denatured by heating, the samples and molecular markers were ready for being loaded into the wells of SDS gel.

1000 ml of 1 x Running Buffer (pH 8.3) was prepared by mixing 100 ml of 10 x Running Buffer with 900 ml of distilled water. To make 1000 ml of 10 x Running Buffer (pH 8.3), 30.3 gram (g) Tris-HCl, 144 g Glycine, and 10 g SDS were dissolved in distilled water and made up to 1000 ml with distilled water.

The whole gel set with glass plates and electrophoresis apparatus were assembled and placed in the electrophoresis tank. The comb was removed from the stacking gel. Both of the assembled electrophoresis apparatus and the wells of the stacking gel were filled up with 1 x Running Buffer. Each sample and 5 µl of pre-stained molecular weight marker were loaded in each well of the stacking gel. The electrophoresis tank was filled up with the rest of 1 x Running Buffer. Samples were run by electrophoresis at 150 Volt for 90 mins, or until the dye molecule (the migration front) migrated to the bottom of the gel.

2.6.4 Blotting/Transfer

1000 ml of 1 x Transfer Buffer, which contained 100 ml of 10 x Transfer Buffer, 700 ml of distilled water and 200 ml of methanol, was prepared. To make 1000 ml of 10 x Transfer Buffer, 30.3 g Tris-HCl and 144 g Glycine were dissolved in distilled water and made up to 1000 ml with distilled water.

The items, including SDS gels, fliter papers, fiber pads and Hybond ECL nitrocellulose membranes (Cat No. RPN203D, GE Healthcare), were pre-soaked in 1 x Transfer Buffer, followed by assembled as a blotting sandwich. The gel faced the negative electrode (black) side and the Hybond ECL nitrocellulose membrane faced the positive electrode (clear) side of the cassettes. A plastic 10 ml tube was rolled over the nitrocellulose membrane to remove bubbles between the membrane and gel. The placing order of each item in the blotting sandwich was shown in Table 2.5. Because proteins were denatured and wrapped in negative charges by SDS, these proteins were migrated from the gel facing negative electrode side to the nitrocellulose membrane facing the positive electrode side during transferring.

Table 2.5. The placing order of each item in the blotting sandwich.

Negative electrode side
Black cassette
1 fiber pad
2-3 sheets of filter papers
SDS gel
1 Hybond ECL nitrocellulose membrane
2-3 sheets of filter papers
1 fiber pad
Clear cassette
Positive electrode side

After the blotting sandwich was assembled, the cassettes were closed and placed into the transblot module. Then, the transblot module, along with an ice pack and magnetic flea, were placed in a tank, which was on a magnetic stirrer. The tank was filled up with 1 x Transfer Buffer and samples were transferred at 100 Volt for 1 hr, or overnight at 20 milliampere (mA).

2.6.5 Visulization of proteins in nitrocellulose membranes

After transferring, the nitrocellulose membrane was washed with 1 x phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST) 3 times (5 mins each time) on an orbital shaker. Then, the nitrocellulose membrane was rinsed in Ponceau-S solution for 5-10 mins on an orbital shaker. The Ponceau-S solution was used to stain proteins and check whether the proteins were transferred on the nitrocellulose membrane. The Ponceau-S was made of 0.1% (w/v) Ponceau S and 5% (v/v) acetic acid in distilled water. The nitrocellulose membrane was destained completely by repeated washing in PBST on an orbital shaker.

2.6.6 Blocking the membrane and incubation with primary antibody

After several washes in PBST, the nitrocellulose membrane was blocked with 5% (w/v) skimmed milk in PBST for at least 1 hr at room temperature on an orbital shaker, in order to prevent non-specific binding of the primary and secondary antibodies. After that, the nitrocellulose membrane was incubated with primary antibody diluted in 5% (w/v) skimmed milk in PBST at 4 ^{o}C, overnight (Table 2.6.). The primary antibodies were diluted at various concentrations, according to manufacturer's recommendation. All antibodies have previously been validated for canine proteins (Paoloni *et al.* 2010).

Table 2.6. Primary antibodies used for western blotting analysis

Protein Name	Manufacturer	Catalog No.	Dilution used	Molecular weight	Raised in	Clonality
PTEN	Abcam	ab23694	1:1000	47	rabbit	polyclonal
phospho (p)-Akt (Ser473)	Cell Signaling	4060	1:1000	60	rabbit	monoclonal
mTOR	Cell Signaling	2972	1:1000	289	rabbit	polyclonal
p-mTOR (Ser2448)	Cell Signaling	2971	1:1000	289	rabbit	polyclonal
p-S6RP (Ser235/236)	Cell Signaling	2211	1:1000	32	rabbit	polyclonal
p-4E-BP1 (Thr37/46)	Cell Signaling	9454	1:1000	15-20	rabbit	polyclonal
p-4E-BP1 (Thr70)	Cell Signaling	9455	1:1000	15-20	rabbit	polyclonal
eIF4E	Cell Signaling	9742	1:1000	25	rabbit	polyclonal
p-eIF4E (Ser209)	Abcam	ab76256	1:1000	25	rabbit	monoclonal
β-actin	Abcam	ab6276	1:5000	45	mouse	monoclonal

2.6.7 Incubation with secondary antibody

After the completion of incubation with primary antibody, the nitrocellulose membrane was washed with PBST three times (5 mins each time) on an orbital shaker. Subsequently, the membrane was incubated with either swine anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody or rabbit anti-mouse HRP conjugated secondary antibody, both of which were purchased from DAKO (Glostrup, Denmark). Both types of secondary antibody were diluted 1:2000 in 5% (w/v) skimmed milk in PBST. The membrane was incubated with secondary antibody for 1 hr on an orbital shaker at room temperature. After that, the membrane was washed in membrane was washed with PBST three times (10 mins each time) on an orbital shaker and prepared for chemiluminescent detection.

2.6.8 Chemiluminescent detection

To detect the presence of a targeted protein band, one volume of a luminol solution which contained luminol (a chemiluminescent substrate for HRP) was mixed with one volume of an enhanced luminol solution. The luminol solution (100 ml) was made up of 1 ml of 250 mM luminol stock, 0.44 ml of 90 mM p-Coumaric acid stock, 6.67 ml of 1.5 M Tris-HCl (pH 8.8), and 91.89 ml of distilled water. The enhanced luminol solution (100 ml) contained 64 µl of 30% hydrogen peroxide, 6.67 ml of 1.5 M Tris-HCl (pH 8.8) and 93 ml of distilled water. Both solutions were protected from light and stored at 4 ^{o}C.

The membrane was covered by the mixing solution composed of the luminol solution and the enhanced luminol solution for 1-2 mins at room temperature. Then, excess solution was drained off. The membrane was transferred to a rectangular glass plate and wrapped in cling film (Saran^{TM} Premiun Wrap). The presence of air bubbles between the cling film and the membrane should be avoided. The wrapped membrane and glass plate were placed in an X-ray film cassette. In a dark room, a sheet of

radiographic film (Hyperfilm ECL, Cat No. 45-001-506, GE Healthcare Amersham) was placed on top of the wrapped membrane and the film cassette was closed for 10 secs to 20 mins, depending on signal intensity. The radiographic film was sent to an automated x-ray film developer for film development.

2.6.9 Stripping

To reuse a blot (nitrocellulose membrane) for detecting and probing other targeted proteins, stripping buffer was used to remove the previous set(s) of protein probes, including primary and secondary antibodies, from the blot. After the film development, the blot (nitrocellulose membrane) was washed with PBST twice (5 mins each time) on an orbital shaker. The blot was rinsed with Restore™ Western Blot Stripping Buffer (Cat No. 21059, Thermo Fisher Scientific Inc-Pierce, USA) for 10-45 mins, depending on the signal intensity of the previous set(s) of protein probes, on an orbital shaker at room temperature. It was recommended that the antibody probed side of the blot faced the stripping buffer, allowing the stripping buffer to work efficiently. After stripping, the stripping buffer was recycled and stored in the dark at room temperature. The blot was washed with PBST 3 times (10 mins each time) on an orbital shaker. To check whether the stripping buffer was compeletly washed away and the proteins remained bound the blot/membrane, Ponceau-S solution was used to stain proteins and the staining method was described in Chapter 2, Section 2.6.3.5. If the proteins on the blot were not stained with Ponceau-S solution, the blot should be washed with PBST until the presence of protein bands with using Ponceau-S staining.

After the blot (membrane) was destained from Ponceau-S completely by repeated washing in PBST on an orbital shaker, the blot was ready for blocking and being reprobed with other primary antibodies by repeating the steps described in Chapter 2, Section 2.6.6 to Section 2.6.9.

2.7 Analysis of drug combination effect

The inhibitory effect of two drug combination on cell viability was defined to be additive, synergistic and antagonistic by using Bliss additivism model. The methods of Bliss analysis was adopted from *Buck E et al* (2006) (Buck *et al.* 2006). Hypothetical curve was generated by using the equation $E_{bliss} = E_A + E_B - (E_A \times E_B)$. While E_A represented the percentage of decreased cell viability by drug A, E_B represented the percentage of decreased cell viability by drug B. Therefore, if the cell decreased viability (%) of the combination of the two drugs experimentally was greater than E_{bliss}, the effect of the combination was considered to be synergistic. On the contrary, if the percentage of decreased viability obtained by an experiment was less than E_{bliss}, the effect of the combination would be considered to be antagonistic. In the present study, the Bliss additivity curves were generated by the combination of various doses of drug A and a constant dose of drug B.

2.8 Statistical Analysis

For analysis of the effects of drugs alone, the values obtained from cell viability assay, as shown in the figures in Chapter 3, were compared with the vehicle control on the same culture plates, then expressed as percentages of mean values with standard deviations (SDs) of at least three replicates using Microsoft Excel 2010 software (Microsoft Corporation, USA).

To analyze the drug combination effects on the inhibition of cell viability, the Anderson-Darling Normality Test was performed on all datasets, in which each dataset contained all values of two drug treatment groups. If the p-value was greater less than 0.05, this indicated that a given dataset fitted the assumption of normal distribution, with 95% confidence, for a t-test. Subsequently, the Two-Sample t-Test with assuming equal variances was performed on the dataset that followed the normal distribution. If the p-

value of the Two-Sample t-Test was less than 0.05, the null hypothesis which stated that the effects of two drug treatment groups were equal was rejected. In other words, the dataset with a p-value less than 0.05 stated that the effects of two drug treatment groups were siginificantly different. If the p-value of the Two Sample t-Test was equal or greater than 0.05, this meant that the effects of the two treatment groups were equal.

For a dataset which did not fit normal distribution (p-value $\leq$0.05), the Mann–Whitney Test was performed on this dataset to determine whether the effects of the two drug treatment groups that were compared in this dataset were significantly different. If the p-value of the Mann–Whitney Test was less than 0.05, the null hypothesis which declared the effects of the two drug treatment groups were the same was rejected. In other words, the effects of the two drug treatment groups that were compared in this dataset were significantly different when p-value <0.05. On the contrary, a dataset with a p-value $\geq$ 0.05, it was declared that both drug treatment groups had the same effects on cell viability inhibition. The Anderson-Darling Normality Test, the Two-Sample t-Test, and the Mann–Whitney Test were performed by using Minitab software (Minitab Inc., State College, PA, USA).

2.9 Microarray

All work of Section 2.9 Microarray coutersy of Dr. Lisa Pang.

2.9.1 RNA isolation

Total RNA from four samples of J3T parental cells and four samples of J3T spheres was purified using the RNA-Bee kit (Qiagen) according to the manufacturer's instructions. Briefly, specimens at a density of 5 x 10^6 cells were homogenized with 1 ml RNA-Bee reagent. Subsequently, phase separation step was performed with addition of 0.2 ml Choloroform (Sigma-Aldrich), followed by placement on ice for 5 mins and, then, centrifuged at 12,000 g (relative centrifugal force (rcf)) for 15 minutes (mins) at 4 ^{o}C. As a result, RNA was separated from cellular components and remained in

transparent aqueous phase. The third step, RNA precipitation, was carried out by transferral of aqueous phase to another tube, followed by addition of 0.5 ml Isopropanol (Sigma-Aldrich), which led to formation of white pellet composed of RNA fragments. Then, the RNA precipitate was washed once with 1 ml 75% ethanol, followed by centrifuged at 7,500 g for 5 mins at 4-25 ^{o}C, air-dried RNA pellet, and resuspended the pellet in ~50 µl of sterile distilled water. The purity of RNA yields was measured by spectrophtometer NanoDrop 6100 (NanoDrop Technologies, DE). The absorbance (A) was measured at 260 nanometer (nm) and 280 nm. The ratio of A_{260}/A_{280} should be within the range between 1.6 and 1.9.

2.9.2 Microarray data analysis

Purified RNA products (1-15 µg of each RNA sample) were sent to ARK-Genomics (the Roslin Institute, University of Edinburgh, UK) for RNA amplification by using MessageAmp aRNA (antisense RNA) kit (Ambion #1750), according to the protocol described on the ARK-Genomics website (http://www.ark-genomics.org/protocols/RNAamplification.php). Subsequently, amplified RNA samples were used to generate Biotin-labeled cRNA templates, which were hybridized to GeneChip Canine 2.0 Genome Arrays (Affymetrix, Santa Clara, CA, USA). Then, the array was washed and scanned with using GeneChip® Operating Software (GCOS) software, according to the protocols described on Affymentrix website (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). The microarray data were analyzed by using Partek Genomic Suite software (Agilent), normalized by using Robust Multichip Averaging (RMA) algorithm. The statistic analysis was performed by using ANOVA with P value set to 0.005 to determine the significant fold changes between the gene profile of progeny cells and that of sphere cells.

2.9.3 Ingenuity Pathway Analysis (IPA)

The microarray data which had gene identifiers with their corresponding fold changes were uploaded to the Ingenuity Pathways Analysis (IPA) database website (http://ingenuity.com/) for mapping each gene identifier to its corresponding gene objective. Graphics of the networks and signaling pathways, which showed genetic expression fold changes, were generated for each data set. In these network graphics, green colour indicated down-regulation (≥1.5 fold change) of a gene expression whereas red colour indicated up-regulation (≥1.5 fold change) of a gene expression.

CHAPTER 3: Dissection of the PI3K/Akt/mTOR pathway identifies potential therapeutic targets in canine tumours

3.1 Abstract

The central role of class I PI3K/Akt/mTOR signaling pathway in the regulation of cell growth, survival, protein synthesis, metabolism and autophagy in normal and cancer cells has made it a promising therapeutic target (Huang and Houghton 2003; Manning and Cantley 2007; Engelman 2009). Using small-molecular inhibitors, the feasibility of the class I PI3K/Akt/mTOR signaling pathway was explored as a therapeutic target in canine oncology either by using pathway inhibitors alone, in combination or combined with conventional chemotherapeutic drugs. The inhibitors used in this study were Wortmannin (pan-class I PI3Ks inhibitor), ZSTK474 (pan-class I PI3K inhibitor), KP372-1 (Akt inhibitor) and Rapamycin (mTOR inhibitor). The results showed that ZSTK474, KP372-1 and Rapamycin significantly down-regulated cell viability at micromolar, nanomolar and micromolar ranges, respectively whereas the effects of Wortmannin were cell line-dependent. Dissection of the mechanism of these inhibitors using western blot analysis and annexin V staining showed that all inhibitors acted to decrease phosphorylation of class I PI3K pathway members. Annexin V staining demonstrated that KP372-1 and Rapamycin induced apoptosis at nanomolar and micromolar ranges respectively whereas the induction of apoptosis by ZSTK474 and Wortmannin were cell-line dependent. Simultaneous inhibition of class I PI3K and mTOR by either Wortmannin or ZSTK474 in combination with Rapamycin additively or synergistically reduced cell viability, with the exception of canine J3T and REM cell lines treated with Wortmannin and Rapamycin. The effects of the four inhibitors combined with the chemotherapeutic agent Doxorubicin were cell line-dependent. Rapamycin-induced autophagy was cell-line dependent. In conclusion, this study shows that the class I PI3K/Akt/mTOR signaling pathway supports the survival and viability of canine tumours and identifies this pathway as a promising therapeutic target.

3.2 Introduction

The class I PI3K/Akt/mTOR signaling pathway comprises a series of serine/threonine kinase cascades that regulate a variety of cellular processes, including cell cycle progression, cellular survival and migration, protein synthesis, motility, metabolism and autophagy. Recent evidence supports the hypothesis that the dysregulation of class I PI3K/Akt/mTOR signaling promotes tumourigenesis and angiogenesis in various cancer types (Altomare and Testa 2005; Chiang and Abraham 2007; Engelman 2009).

The class I PI3K/Akt/mTOR pathway is frequently activated by two types of cell surface receptors, which are RTKs and GPCRs after binding to their ligands such as growth factors, hormones and chemokines (Dorsam and Gutkind 2007; Lemmon and Schlessinger 2010). The class I PI3Ks can be further categorized into class IA PI3K and class IB PI3K, according to their upstream regulators and regulatory subunits. The RTKs stimulate class IA PI3Ks by direct interaction with p85 regulatory subunit and indirect interaction with p110α/p110β/p110δ catalytic subunit of class IA PI3K via Ras. The GPCRs stimulate class IB PI3K through interaction with p110γ catalytic subunit via Ras and with p101 or p87/p84 regulatory subunit of class IB PI3K (Wymann and Pirola 1998; Suire *et al.* 2005). After class I PI3Ks are activated, their catalytic subunits can transfer the γ-phosphate group of ATP to PtdIns(4,5)P_2, leading to the conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Subsequently, the PtdIns(3,4,5)P_3 can recruit PDK1 and Akt to the inner side of cell membrane, which facilitates PDK1 to phosphorylate and activate Akt. In contrast, Akt activity can be counteracted by PTEN through conversion of PtdIns(3,4,5)P_3 back to PtdIns(4,5)P_2 (see Chapter 1, Figure 1.01) (Faivre *et al.* 2006).

Activated Akt can transduce signals to mTORC1 which is composed of mTOR, Raptor, PRAS40 and mLST8 (Sancak *et al.* 2007; Vander Haar *et al.* 2007; Ramirez-Valle *et al.* 2010). Akt activates mTORC1 through two routes. The first route is that Akt inhibits TSC1–TSC2 complex by direct phosphorylation of TSC2. This decreases

TSC1–TSC2 complex-regulated GAP activity towards Rheb and in turn maintains high levels of active GTP-bound Rheb. The GTP-bound Rheb acts as a positive regulator of mTORC1 (Manning *et al.* 2002; Manning and Cantley 2003). Alternatively, Akt phosphorylates PRAS40 and impairs PRAS40-mediated mTORC1 inhibition (see Chapter 1, Figure 1.01) (Sancak *et al.* 2007; Vander Haar *et al.* 2007).

The class I PI3K effects on cellular functions through its two major downstream effectors Akt and mTOR. The activated Akt can perform multiple functions by direct phosphorylation of its downstream effectors. These functions of the Akt are activation of protein synthesis through mTORC1, promotion of G1/S cell cycle progression through the substrates such as $p21^{WAF1}$, $p27^{KIP}$ and GSK3, promotion of G2/M cell cycle progression through Chk1, against apoptosis through FoxO transcription factors and Bad, promotion of survival through IKK-α and MDM2, encouragement of glucose uptake into cell through AS160, up-regulation of glycogen synthesis through GSK-3, and inhibition of gluconegesis through FoxO1 (see Chapter 1, Figure 1.08) (Cross *et al.* 1995; Datta *et al.* 1997; Brunet *et al.* 1999; Ozes *et al.* 1999; Nakae *et al.* 2001; Zhou *et al.* 2001; Liang *et al.* 2002; Huang and Houghton 2003; Eguez *et al.* 2005; Puc *et al.* 2005; Patel *et al.* 2008; Fenouille *et al.* 2011; van der Vos and Coffer 2011).

mTORC1 plays an important role in regulation of protein synthesis via its two well-characterized downstream effectors, p70S6K and 4E-BP1. mTORC1 activates p70S6K through direct phosphorylation of p70S6K on Thr229, Thr389, Ser404, and Ser411. This enables p70S6K to activate S6RP through phosphorylation of S6RP on Ser235, Ser236, Ser240, and Ser244, which results in triggering S6RP-modulated translation of TOP mRNAs that frequently encode ribosomal proteins and elongation factors (see Chapter 1, Figure 1.01) (Ferrari *et al.* 1991; Terada *et al.* 1994; Han *et al.* 1995; Jefferies *et al.* 1997; Iadevaia *et al.* 2008). mTORC1 phosphorylates 4E-BP1 on Thr37, Thr46, Thr70 and Ser65 in a hierarchical manner, which causes the dissociation of eIF4E from 4E-BP1 and allows Mnk1/2 to phosphorylate eIF4E on Ser209. Subsequently, the free and/or phosphorylated eIF4E enters an eIF4E complex which is composed of eIF4A, eIF4E and eIF4G. The assembly of the eIF4F complex activates

translation of mRNA with 5' terminal cap (see Chapter 1, Figure 1.01) (Raught and Gingras 1999; Mothe-Satney *et al.* 2000). The 4E-BP1/eIF4E-modulated cap-dependent translation can synthesize proteins that promote cell growth (e.g. cyclin D1 and c-Myc) and neovascularization (e.g. VEGF, bFGF) and tumour-associated malignancies (e.g. matrix metalloprotease 9) (De Benedetti and Graff 2004; Mamane *et al.* 2004).

mTORC1 is not only the signaling downstream of RTKs and GPCRs in response to their ligands such as growth factors and chemokines but also acts as a sensor of nutrient and energy status and stress stimulus. Low levels of energy, nutrient deprivation, stress stimuli or decreased the availability of growth factors down-regulates mTORC1 activity, resulting in down-regulation of protein synthesis but up-regulation of autophagy (see Chapter 1, Figure 1.09) (Jung *et al.* 2010). Autophagy is a cellular process for cells to efficiently utilize limited energy sources and maintain cell survival (He and Klionsky 2009). mTORC1 acts as negative regulator of autophagy through phosphorylation of ULK1/2 and Atg13 (see Chapter 1, Figure 1.10) (Yan *et al.* 1999).

In addition to mTORC1, mTOR can be in complex with Rictor, mSin1, Protor-1/2 and mLST8 to form mTORC2 (Frias *et al.* 2006; Yang *et al.* 2006; Pearce *et al.* 2007; Zoncu *et al.* 2011). In contrast to mTORC1 that acts as Akt substrate, mTORC2 acts as upstream activator of Akt. mTORC2 activates Akt by direct phosphorylation of Akt on Ser473 (see Chapter 1, Figure 1.01) (Huang and Houghton 2003).

Over the past two decades, a large body of literature has shown that overexpression of the class I PI3K/Akt/mTOR pathway is tightly correlated with tumourigenesis. The constitutive activation of PI3K kinase cascades can be instigated by molecular alterations in components of the class I PI3K/Akt/mTOR axis pathway and its upstream signals. So far, molecular alterations or post-translational modifications in RTKs such as KIT and EGFR, Ras, class I PI3K, PTEN, Akt, TSC1-TSC2 complex, p70S6K, 4E-BP1, eIF4E and Rictor (an mTORC2 component) have been identified in human cancers (Wong *et al.* 1992; Sakai *et al.* 1998; Sorrells *et al.* 1999; Niida *et al.* 2001; Bellacosa *et al.* 2005; Akin 2006; Masri *et al.* 2007; Heinonen *et al.* 2008; Silva *et*

al. 2008; Karlsson *et al.* 2010; Castellano and Downward 2011). Alterations of RTKs such as KIT and EGFR, Ras and PTEN have been identified in canine tumours (Levine *et al.* 2002; Richter *et al.* 2005; Gleixner *et al.* 2007; Gama *et al.* 2009).

In human oncology, many small molecular inhibitors that target one or two components of the class I PI3K/Akt/mTOR pathway are available for preclinical studies and clinical trials for cancer therapy. So far, Temsirolimus and Everolimus, both of which are Rapamycin analogs and target mTOR kinase, have been approved by FDA for monotherapy in patients with either advanced renal cell carcinomas (Temsirolimus and Everolimus) or progressive neuroendocrine tumors of pancreatic origin (Everolimus) (http://www.cancer.gov/)(Kwitkowski *et al.* 2010; Saif 2011). In veterinary oncology, a phase I trial study of Rapamycin monotherapy in patients with canine osteosarcoma showed that the drug doses tested in this study were well-tolerated in patients (Paoloni *et al.* 2010).

The aim of this study was to investigate the role of class I PI3K/Akt/mTOR pathway in tumourigenicity of canine cancers *in vitro*. Four small molecular inhibitors targeting class I PI3K, Akt, or mTOR was used to treat five cell lines derived from different canine cancers to evaluate the drug efficacy in cell viability inhibition and to investigate the mechanism of these inhibitors. To investigate effects of the combination therapy on these canine lines, cells were treated with either the combination of two inhibitors or the combination of the inhibitors with Doxorubicin.

3.3 Results

3.3.1 Class I PI3K signaling is activated in canine cancer cells

To determine the PI3K kinase activity in the five canine tumour cell lines (C2, J3T, REM134, SB and 3132), Western blot analysis (see Chapter 2, Section 2.6) was employed to examine active (phosphorylated) forms of several components of PI3K pathway, including phosphorylated Akt, mTOR, S6RP, 4E-BP1 and eIF4E. In addition

to these canine cells, the human Jurkat T leukemic cell line was used as control as the cell line has constitutive activation of PI3K signaling through PTEN loss (Shan *et al.* 2000). As shown in Figure 3.01, all canine cell lines with either PTEN expression (3132, SB, J3T and C2 cells) or PTEN loss (REM134 cells) expressed detectable levels of active forms of these proteins, indicating active PI3K signaling in these canine cells. It was observed that all cell lines expressed three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α). The hyperphosphorylated isoform (γ) is phosphorylated with mTORC1 with high affinity and does not bind to eIF4E whereas the unphosphorylated isoform (α) strongly binds eIF4E. The middle form (β) is phosphorylated by mTORC1 and interacts with eIF4E with low affinity (Lin *et al.* 1995; Beretta *et al.* 1996; Gingras *et al.* 1996; Gingras *et al.* 1998).

Because accumulating evidence suggests cross-talk between PI3K and Ras/Raf/Erk MAPK pathways occurred frequently, the activity of the Erk-MAPK pathway was investigated in these canine cells (Aksamitiene *et al.* 2010; Andreu-Perez *et al.* 2010; Shao and Aplin 2010). The Western blot results showed that the canine cells expressed detectable levels of active (phosphorylated) forms of ERK1/2, indicating Ras/Erk MAPK signaling is activated in these canine cells (Figure 3.01).

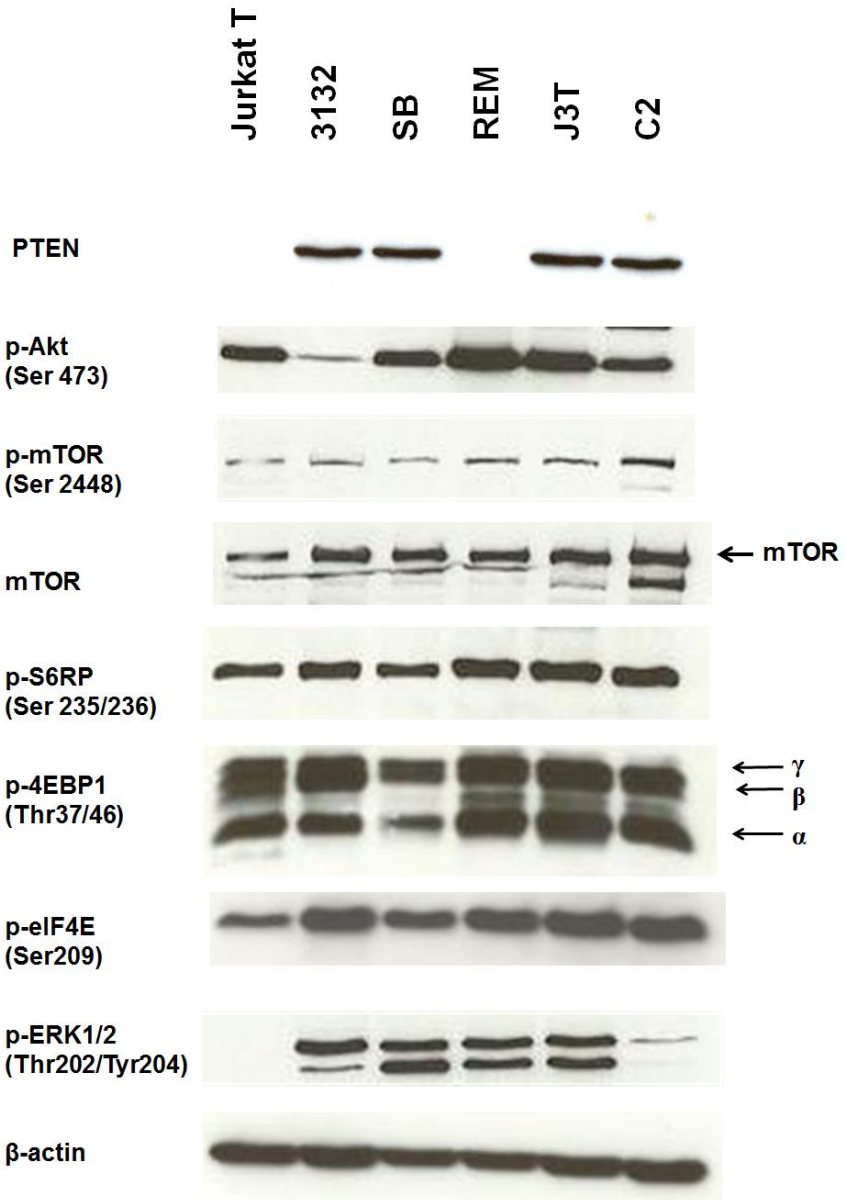


Figure 3.01. Western blot analysis of components of the class I PI3K and ERK pathways in human and canine cancer cells. Whole cell lysates comprising 50 µg total protein were subjected to Western blotting analysis (see Chapter 2, Section 2.6) with β-actin as a loading control. Three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α), were observed (Gingras *et al.* 1998).

3.3.2 Effects of the inhibitors targeting class I PI3K/Akt/mTORC1 axis pathway

3.3.2.1 Effects of inhibitors on cell viability

To investigate the potential central role of PI3K signaling in cells, specific chemical inhibitors were used to block pathway components. Inhibitors used were Wortmannin (pan-class I PI3K inhibitor), ZSTK474 (pan-class I PI3K inhibitor), KP372-1 (Akt inhibitor) and Rapamycin (mTORC1 inhibitor). Subsequently, cell viability of drug-treated cells was compared with those of vehicle-treated cells by using a standard cell viability assay (see Chpater 2, Section 2.3 and Section 2.4). As shown in Figure 3.02A, the responses of these cell lines to Wortmannin were variable. At the highest dose (40 μM), canine C2 cells as well as human Jurkat T control cells were the most sensitive to Wortmannin, which reduced cell viability by >80%. 3132 and SB cells were relatively sensitive to Wortmannin with inhibition of ~50% cell viability. J3T and REM134 cells showed relative resistance to Wortmannin with less than 20% inhibition of cell viability. It was previously reported that Wortmannin selectively inhibited class I and class II PI3K (except PI3KC2α) at a similar potency. In addition, Wortmannin also targeted PI3KC2α and other serine/threonine kinases that contain catalytic domains resembling PI3Ks, such as mTOR, ATM, DNA-PK and phosphatidylinositol 4-kinase (PI4K). But the doses used for inhibition of these PI3K-related kinases were at least 10-fold higher than those for class I PI3K inhibition (Nakanishi *et al.* 1995; Domin *et al.* 1997; Banin *et al.* 1998; Misawa *et al.* 1998; Izzard *et al.* 1999). Compared with Wortmannin, the novel pan-class I PI3K inhibitor was more specific to class I PI3K and was reported to have weak or no inhibitory effect on other Wortmannin targets (Kong and Yamori 2007; Kong *et al.* 2009; Kong *et al.* 2010). In this study, ZSTK474 at 10 μM effectively down-regulated ≥74% cell viability in all lines and fully inhibited the viability of SB (96%) and Jurkat T cells (100%). ZSTK474 at concentrations between 10 μM and 40 μM resulted in a slower decline in J3T, C2 and 3132 cells and no further inhibition in REM134 and SB cells (Figure 3.02B).

KP372-1, an Akt inhibitor, efficiently inhibited all cell lines causing 100% loss of cell viability after incubation with this compound at concentrations of ≥ 250 nM for 2 days, compared with the other three inhibitors, which required a longer period of time (3 days) and much higher doses (micromolar concentrations) to reach effective inhibition (Figure 3.02). Notably, REM134 cells were most sensitive to KP372-1 with full inhibition of cell viability at concentrations ≥ 62.5 nM.

With regard to Rapamycin, it was observed that the doses within a nanomolar range had limited inhibitory effects on the viability of these canine cells. Jurkat T cells were observed to be most sensitive to Rapamycin, with an IC-50 (concentration for 50% inhibition of viability of ~ 1nM). By contrast, all canine cancer cell lines were relatively resistant to Rapmycin with IC50 values for 3132, C2, SB, REM134 and J3T cells of 1 µM, 1-10 µM, 10 µM, 10-20 µM and > 20 µM, respectively. Canine J3T and REM134 cells were most resistant to Rapamycin. The doses for Rapamycin to reach full inhibition of all lines were between 20 µM and 40 µM (Figure 3.02D).

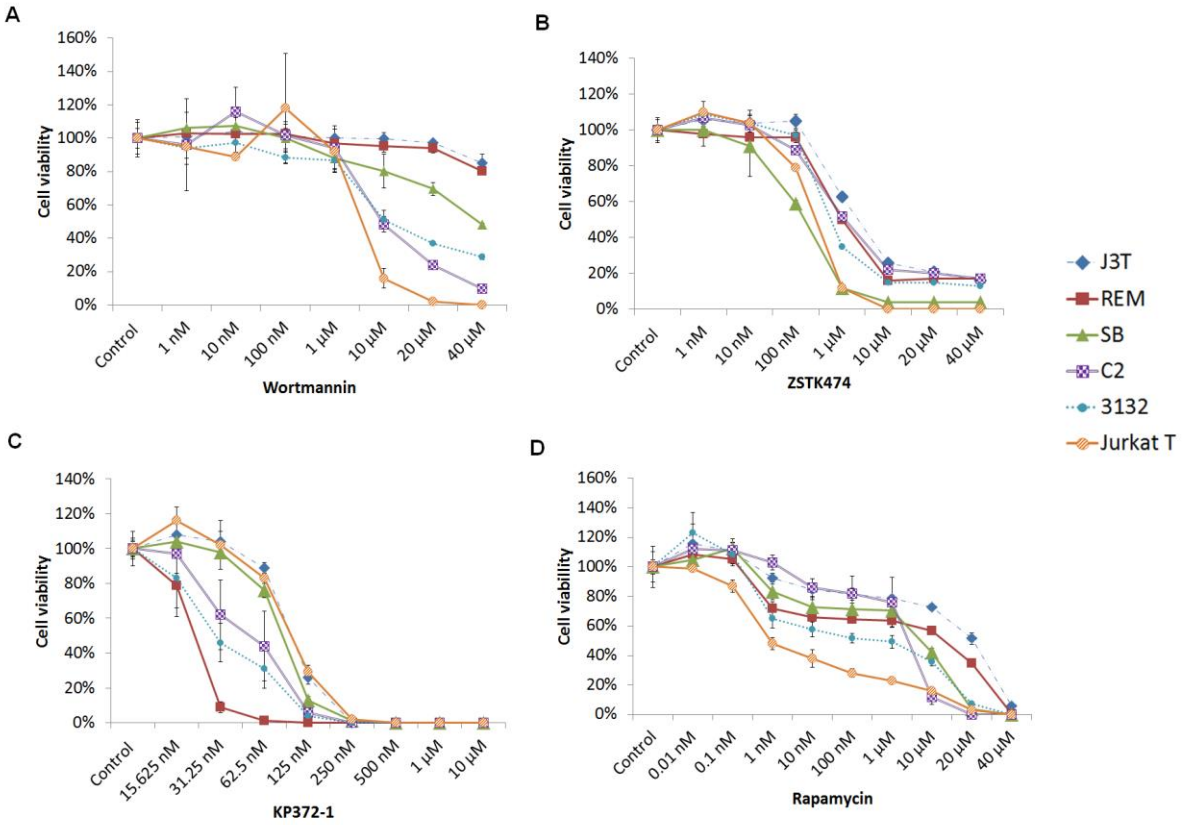


Figure 3.02. Sensitivity of canine and human cancer cells to inhibitors targeting class I PI3K/Akt/mTOR pathway. Cells were treated with a range of doses of the pan-class I PI3K inhibitors Wortmannin for 3 days (A), ZSTK474 for 3 days (A), Akt inhibitor KP372-1 for 2 days (B), or mTOR inhibitor Rapamycin for 3 days (C) (see Chpater 2, Section 2.3). After drug treatment, the number of viable cells was determined by using CellTiter-Glo® Luminescent Cell Viability Assay (see Chapter 2, Section 2.4). Results are expressed as mean (±standard deviations (SD)) counts of quadruplicate wells, relative to vehicle (DMSO)-treated controls on the same culture plates.

3.3.2.2 Effects of the inhibitors on PI3K signaling by Western Blotting

To study the inhibitory effects of Wortmannin, ZSTK474, KP372-1 and Rapamycin on the PI3K/Akt/mTOR axis signaling in cells, Western blot analysis (see Chapter 2, Section 2.6) was performed to evaluate expression levels of active (phosphorylated) forms of class I PI3K downstream effectors, including Akt, S6RP, 4E-BP1 and eIF4E. Previous studies demonstrated that 10-100 nM Wortmannin, 0.5-2 μM ZSTK474, and 10 nM Rapamycin could reduce the phosphorylation levels of components of the class I PI3K/Akt/mTOR axis pathway in a variety of cancer cell lines (Yaguchi *et al.* 2006; El-Salem *et al.* 2007; Howes *et al.* 2007). As for KP372-1, the doses of inhibitory concentration of 100% cell viability (IC-100) and lower than IC-100 were used to treat cancer cells, which were cultured in serum-containing medium, for 8-16 hours (Zeng *et al.* 2006). Therefore, in this study, 1 μM Wortmannin and ZSTK474, 100 nM Rapamycin and the concentration of IC-100 of KP372-1, were used to investigate the inhibitory of effect on the class I PI3K/Akt/mTOR axis pathway in all cell lines.

As shown in Figure 3.03A, Wortmannin down-regulated phosphorylation levels of Akt in most cell lines but not in REM134 or J3T cells, indicating this inhibitor abrogated PI3K activity only in Jurkat T, 3132, SB and C2 cells. Compared with Wortmannin, ZSTK474 showed more potent effects on PI3-Kinase inhibition, based on down-regulated phosphorylation of Akt and even mTORC1 substrates S6RP and 4EBP1. Both PI3K inhibitors were observed to slightly up-regulate eIF4E phosphorylation in Jurkat T cells (Figure 3.03A and 3.03B).

KP372-1, at 400 nM, down-regulated phosphorylation levels of S6RP and 4E-BP1 in all cell lines. Moreover, it profoundly inhibited phosphorylation of Akt and eIF4E in J3T and REM134 cells. However, in Jurkat T cells, the phosphorylation of eIF4E was significantly up-regulated (Figure 3.03C).

Rapamycin inhibited mTORC1 signaling in all cell lines, based on decreased hyperphosphorylation levels of 4EBP1 isoform (γ) and/or phosphorylation of S6RP

(Figure 3.03A). As for the effects of Rapamycin on three 4EBP1 isoforms, this inhibitor was observed to decrease the levels of hyperphosphorylated γ isoform, which was phosphorylated with mTORC1 with high affinity and did not bind eIF4E. However, Rapamycin did not decrease the levels of middle β isoform and unphosphorylated α isoform. The middle β isoform of 4EBP1 was phosphorylated by mTORC1 and interacted with eIF4E with low affinity. The unphosphorylated α isoform of 4EBP1 stronly bound eIF4E (Lin *et al.* 1995; Beretta *et al.* 1996; Gingras *et al.* 1996; Gingras *et al.* 1998).

Instead of down-regulation of eIF4E phosphorylation, Rapmaycin up-regulated eIF4E activity in Jurkat T and SB cells (Figure 3.03A).

To investigate whether the effects of Rapamycin on S6RP, 4EBP1 and eIF4E phosphorylation were transient or long-term, prolonged exposure of cells to this inhibitor was performed at two different time points, 48 and 72 hours. As shown in Figure 3.04, the effects of Rapamycin on mTORC1 signaling at 48 and 72 hours were the same as those at 5 hours in all cell lines.

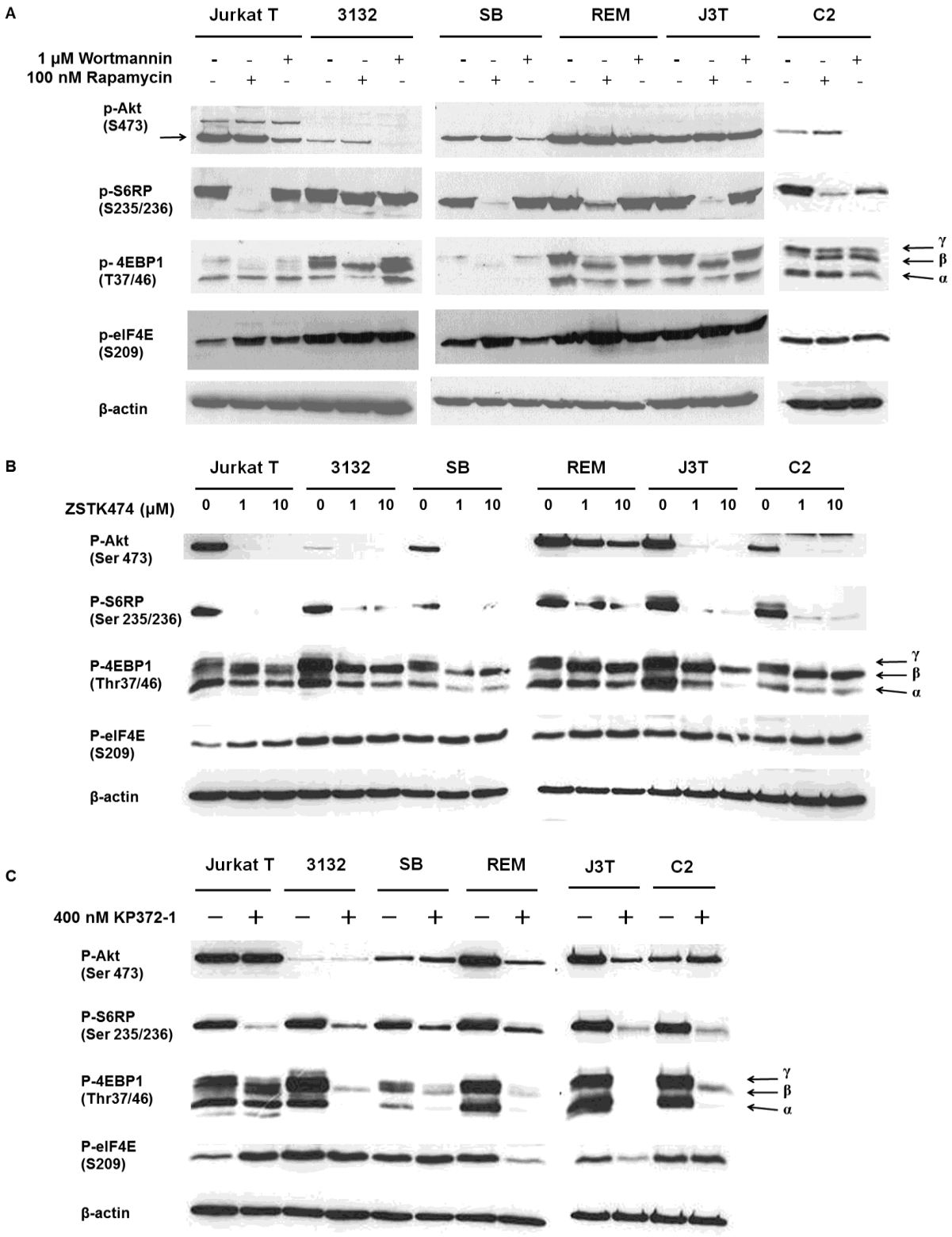

A
Jurkat T
3132
SB
REM
J3T
C2
1 μM Wortmannin
100 nM Rapamycin
p-Akt (S473)
p-S6RP (S235/236)
p-4EBP1 (T37/46)
p-eIF4E (S209)
β-actin
γ
β
α
B
Jurkat T
3132
SB
REM
J3T
C2
ZSTK474 (μM)
0 1 10 0 1 10 0 1 10 0 1 10 0 1 10 0 1 10
P-Akt (Ser 473)
P-S6RP (Ser 235/236)
P-4EBP1 (Thr37/46)
P-eIF4E (S209)
β-actin
γ
β
α
C
Jurkat T
3132
SB
REM
J3T
C2
400 nM KP372-1
P-Akt (Ser 473)
P-S6RP (Ser 235/236)
P-4EBP1 (Thr37/46)
P-eIF4E (S209)
β-actin
γ
β
α

Figure 3.03 Effects of the inhibitors on class I PI3K/Akt/mTOR axis signaling in canine and human cancer cells. Cells were seeded at a density of 20,000 cells/ml and incubated overnight, followed by treatment with 1μM Wortmannin, 100 nM Rapamycin (A), 1 μM or 10 μM ZSTK474 (B), or 400 nM KP372-1 (C) for 5 hours (see Chapter 2, Section 2.3). Whole cell lysates, comprising 50 μg total protein, were subjected to Western blot with the indicated antibodies (see Chapter 2, Section 2.6). β-actin was used as a loading control. The arrows indicated the targeted protein bands recognized by primary antibodies. Three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α), were observed (Gingras *et al.* 1998).

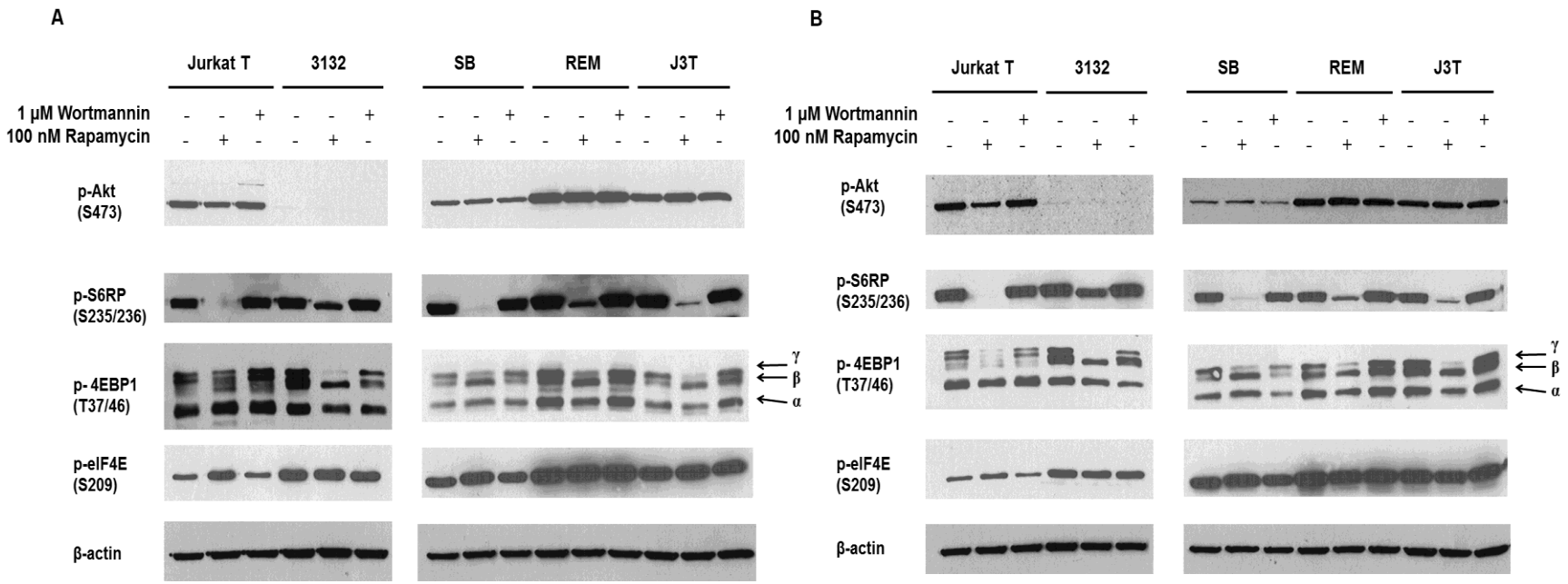


Figure 3.04 Rapamycin increases eIF4E phosphorylation in Jurkat T and SB cells. Cells were seeded at a density of 20,000 cells/ml and incubated overnight, followed by treatment with 1 μM Wortmannin, 100 nM Rapamycin or vehicle control for 48 hours (A) and 72 hours (B) (see Chapter 2, Section 2.3). Whole cell lysates (comprising 50 μg total protein) were subjected to Western blot with the indicated antibodies (see Chapter 2, Section 2.6). β-actin was used as a loading control. Three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α), were observed (Gingras *et al.* 1998).

3.3.2.3 Time course study of drug efficacy by Western blotting technique

To dissect the dynamics of inhibition further, a time-course study was performed utilizing the C2 cell line. As shown in Figure 3.05A, both pan-PI3K inhibitors ZSTK474 and Wortmannin blocked PI3K activity, as evidenced by significant reduction in phosphorylation levels of Akt and its downstream substrates S6RP and γ hyper-phosphorylated form of 4E-BP1. However, compared with Wortmannin, ZSTK474 showed greater potency and longer efficacy in down-regulating PI3K kinase signaling, based on the results showing that inhibition of phosphorylation of downstream elements of PI3K by ZSTK474 lasted for 50 hours whereas Wortmannin lasted for 12 hrs (Figure 3.05A).

For the time course study of KP372-1 in C2 cells, three doses higher than inhibitory concentration of 100% cell viability (IC-100), including 150, 200 and 400 nM, were tested. On one hand, the highest dose (400 nM) decreased the phosphorylation levels of PI3K/Akt substrates S6RP and 4EBP1 at the earlier time point of 4 hours but inhibited profoundly the phosphorylation of all PI3K substrates, including Akt, S6RP, 4E-BP1 and eIF4E, at later time points of 8 and 12 hours (Figure 3.05B). On the other hand, KP372-1 at concentrations between 150 nM and 200 nM showed no inhibitory effects on PI3K activity at early time points (4 and 8 hours) but gradually down-regulated all of its downstream components at later time points (12, 21 and 24 hours). Results for treatment with 200 nM and 400 nM KP372-1 at later time points (21 and 24 hours) were unavailable, due to these cell lysates with little amount of or no protein (Figure 3.05B). It was assumed that these cell lysates with scarce amount of protein were related to the late apoptosis (or secondary necrosis) of the majority of C2 cells, which cause leakage of proteins to cell medium (Figure 3.06C) (Krysko *et al.* 2008).

As shown in Figure 3.04 and 3.05A, indicators of inhibition of mTORC1 signaling by Rapamycin, such as down-regulation of S6RP and γ form of 4E-BP1 phosphorylation, were evident for 72 hours. This is consistent with previous findings which confirms that the efficacy of Rapamycin can last for ~3 days (Faivre *et al.* 2006).

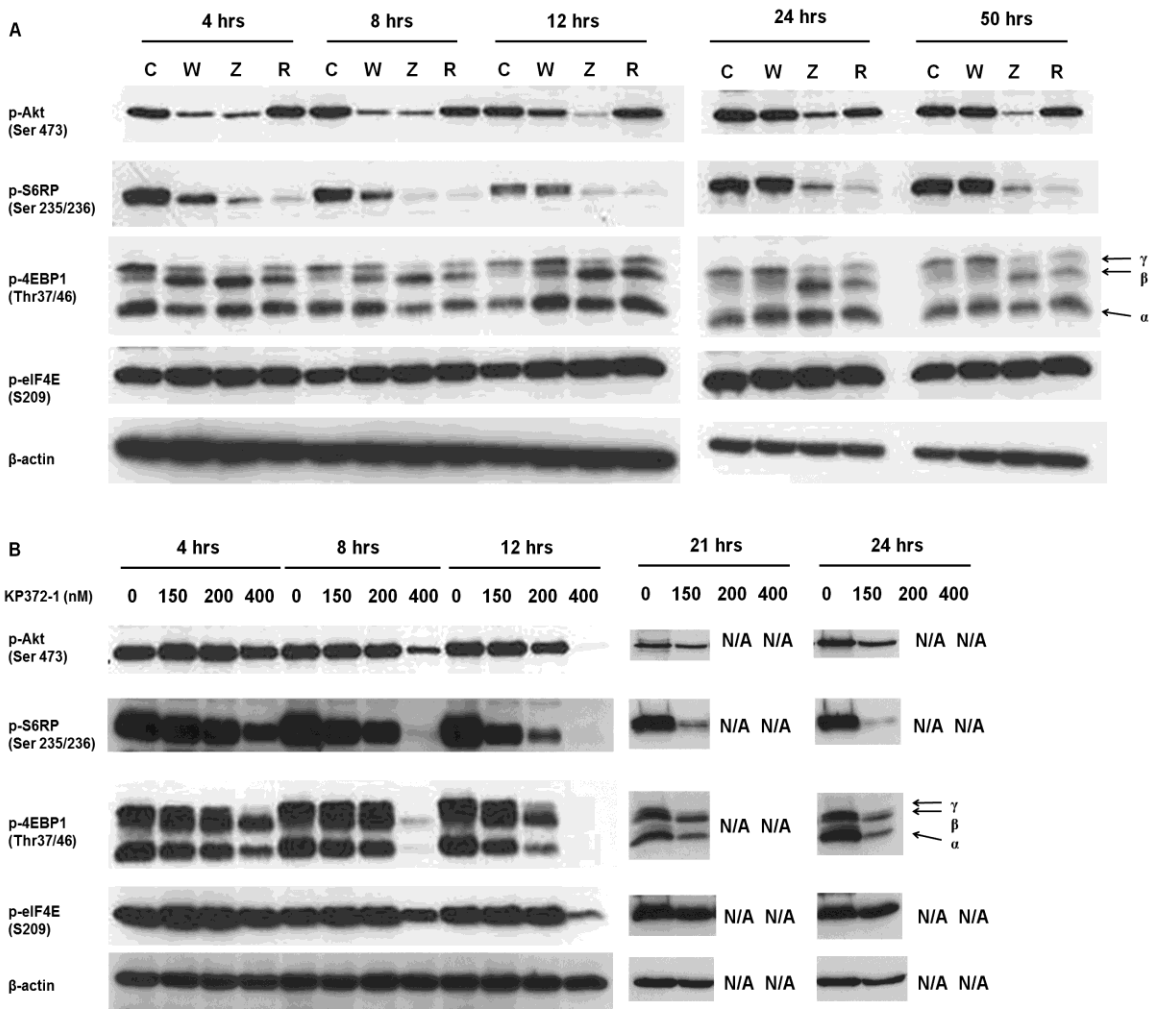


Figure 3.05. Effects of the inhibitors on class I PI3K/Akt/mTOR axis signaling in canine C2 cells. Cells were treated with vehicle control DMSO (C), pan-class I PI3K inhibitor Wortmannin (W) at 1 μM and ZSTK474 (Z) at 1 μM, mTOR inhibitor Rapamycin (R) at 100 nM (A), and Akt inhibitor KP372-1 at 0, 150, 200 and 400 nM (B) for the indicated period of time. Whole cell lysates (comprising 50 μg total protein) were subjected to Western blot with the indicated antibodies (see Chapter 2, Section 2.6). β-actin was used as a loading control. N/A indicates data unavailable due to induction of apoptosis in all cells. Three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α), were observed (Gingras *et al.* 1998).

3.3.2.4 Effects of PI3K/Akt/mTOR inhibitors on apoptosis induction

To determine whether the four PI3K pathway inhibitors Wortmannin, ZSTK474, KP372-1 and Rapamycin induce apoptosis, cells were stained with annexin V (a cell apoptosis marker) and propidium iodide (PI) (taken up by dead and necrotic cells), followed by flow cytometry (see Chapter 2, Section 2.5).

3.3.2.4.1 Utilization of C2 cells to titrate drug dosage

Using C2 cells to titrate these four PI3K pathway inhibitors, KP372-1 was observed to induce apoptosis of the majority of cells, increasing cell loss by 71% and 76% at 200 nM and 400 nM, respectively (Figure 3.06). The other three inhibitors Wortmannin, ZSTK474, and Rapamycin induced significant apoptosis at concentrations greater than 10 μM. At 10 μM, Wortmannin, ZSTK474, and Rapamycin increased apoptosis by 39%, 19% and 20% respectively, as compared with the controls. Notably, the apoptosis rates were measured after cells were exposed to KP372-1 for 1 day whereas the exposure period for the other drugs was 2 days, suggesting KP372-1 as the most potent inducer of apoptosis.

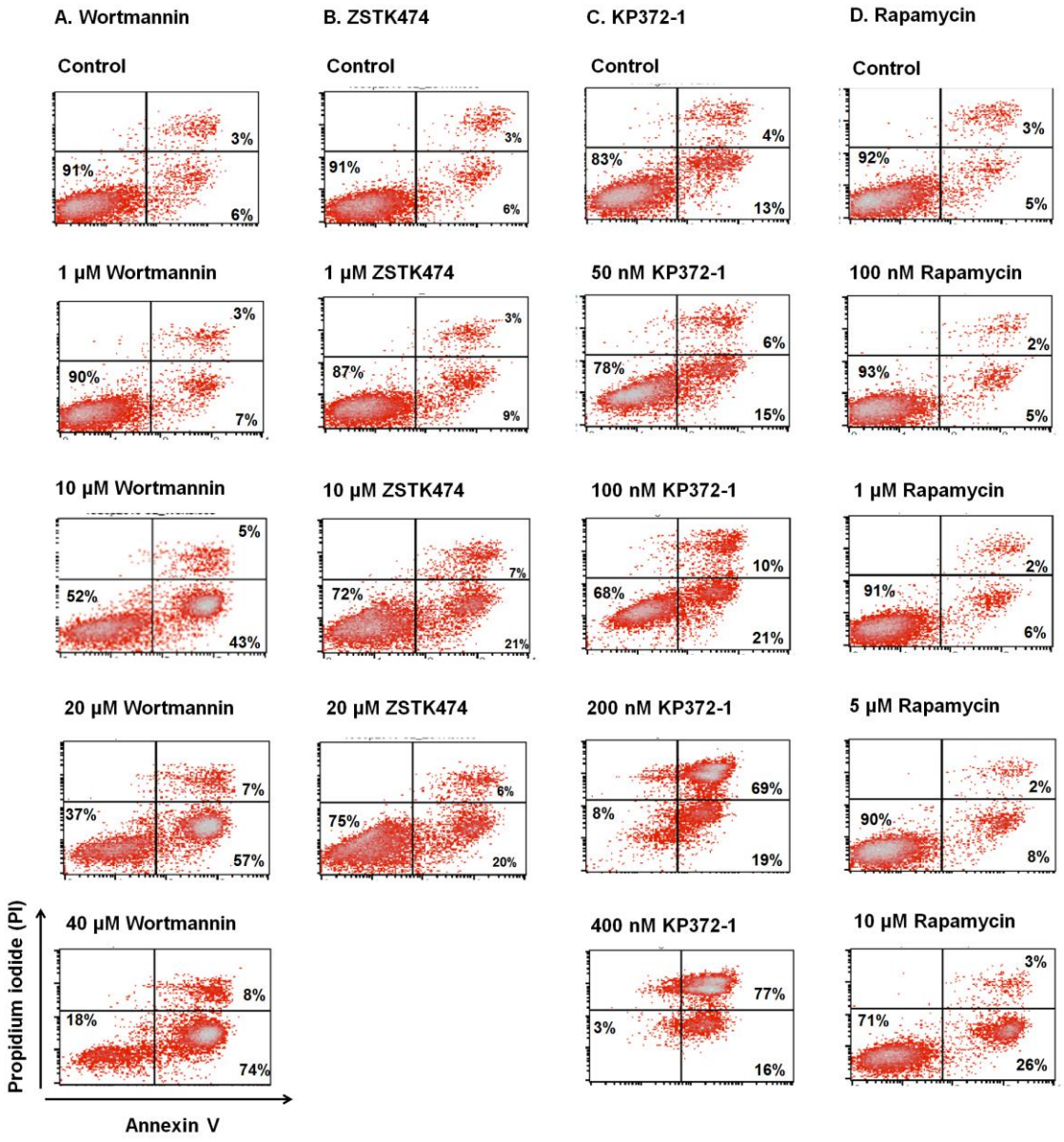


Figure 3.06. Effects of the class I PI3K pathway inhibitors on apoptosis of canine C2 cells. The C2 cells were treated with the indicated concentrations of Wortmannin, ZSTK474, and Rapamycin for 2 days, and of K P372-1 for 1 day. Induction of apoptosis was determined by measuring/quantifying annexin V/PI staining using flow cytometry (see Chapter 2, Section 2.5). Live, early apoptotic and late apoptotic cells were located, respectively, in the lower left, lower right and upper right quadrants of a bivariate dotplot.

3.3.2.4.2 Effects of the class I PI3K/Akt/mTOR inhibitors on apoptosis of all cancer cell lines

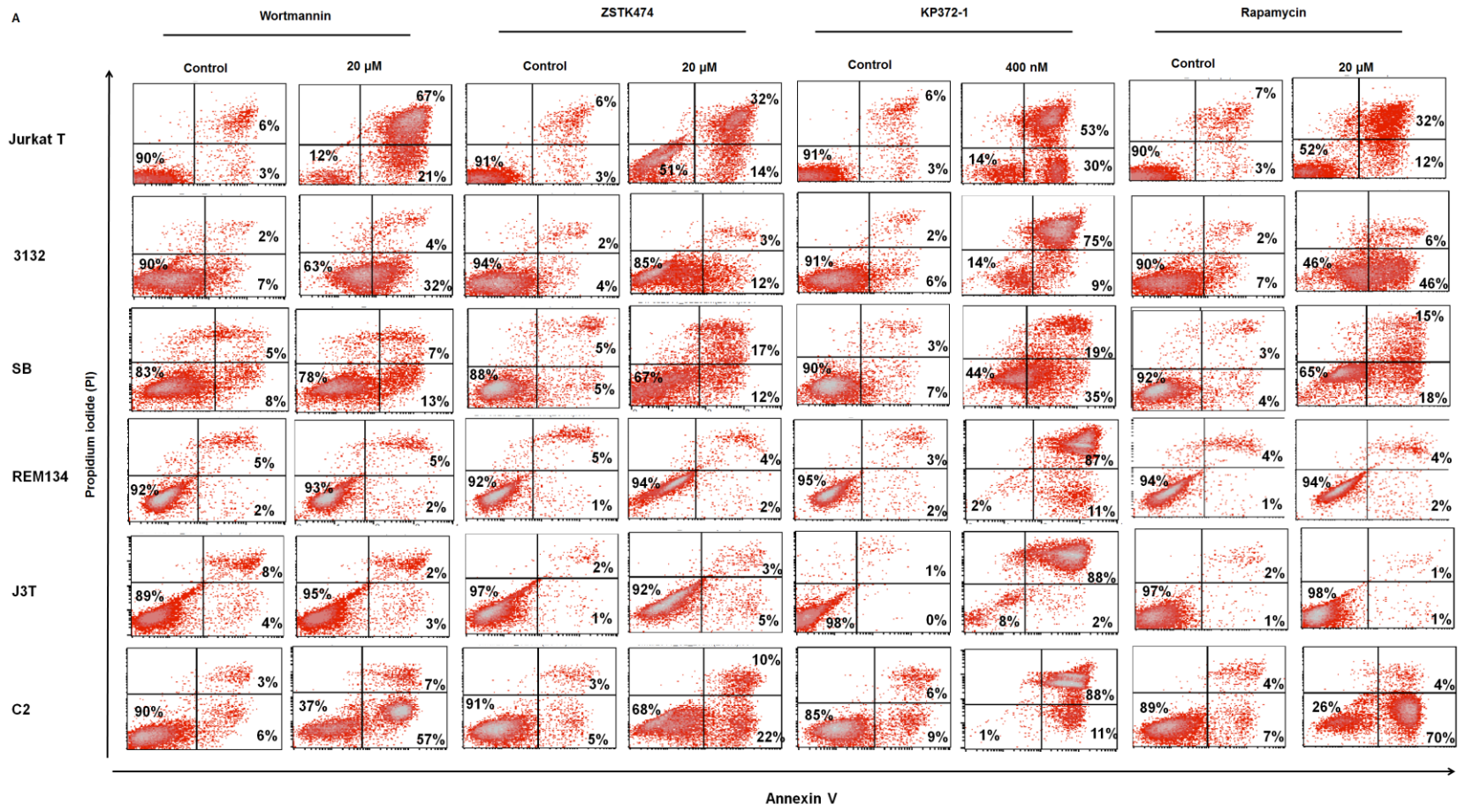
All cell lines were treated with Wortmannin, ZSTK474 and Rapamycin at 20 μM for 2 days and with KP372-1 at 400 nM for 1 day. The data obtained from two individual experiments showed that Wortmannin significantly increased apoptosis of Jurkat T cells by 79% and 68% in 1st and 2nd experiments respectively, C2 by 55% and 28%, and 3132 by 27% and 17% as compared with controls. However, Wortmannin did not have any obvious efficacy on the other cell lines (Figure 3.07A and 3.07B).

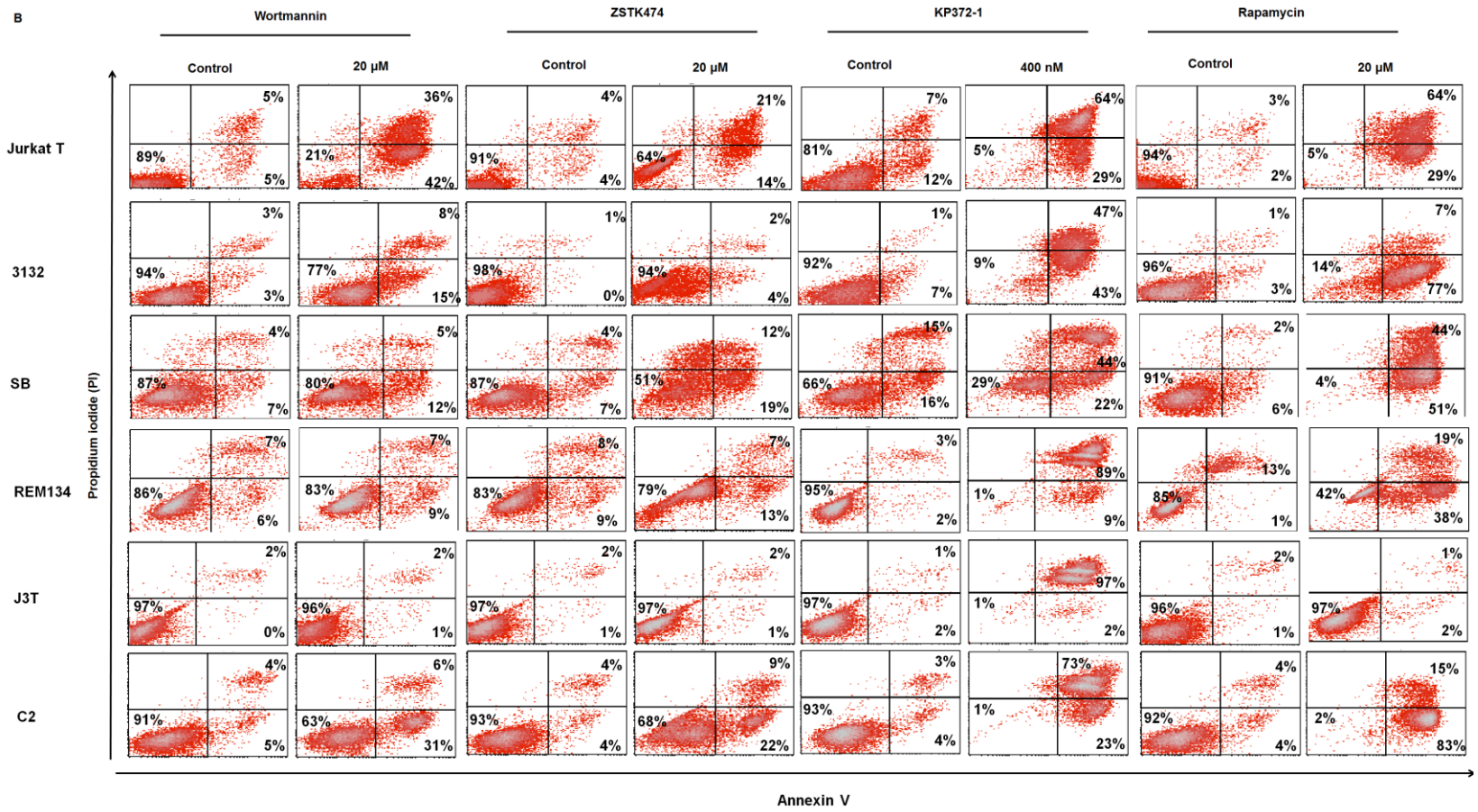
ZSTK474 significantly increased apoptosis of Jurkat T cells by 35% and 27% in 1st and 2nd experiment respectively, C2 by 24% and 23%, and SB by 19% and 20%, as compared with the controls. By contrast, ZSTK474 showed no efficacy towards the other cell lines (Figure 3.07A and 3.07B).

KP372-1 increased apoptosis of Jurkat T cells by 74% and 74% in 1st and 2nd experiments respectively, 3132 by 76% and 82%, SB by 44% and 35%, REM134 by 93% and 93%, J3T by 89% and 96% and C2 by 84% and 89% (Figure 3.07A and 3.07B).

With regard to Rapamycin, the data obtained from the 1st experiment showed that 20 μM Rapamycin significantly increased apoptosis of all cell lines by 26%-62%, except REM134 and J3T cells (Figure 3.07A). In the 2nd experiment, Rapamycin at the same concentration showed better efficacy in apoptosis induction with an increase in apoptotic fraction by 80%-95% in most cell lines (including Jurkat T, 3132, SB and C2 cells) and by 43% in REM134 cells. In contrast, no apoptosis was observed in Rapamycin-treated J3T cells (Figure 3.07B). Due to the inconsistent results from REM134 cells, the 3rd experiment was performed on this cell line and the data showed that Rapamycin at the concentration of 20 μM increased apoptosis by 31%, as compared with the control (Figure 3.07C). Taken together, it was observed that Rapamycin had no effect on inducing apoptosis of REM cells in the 1st experiment whereas this inhibitor increased apoptosis of the same cell line in the 2nd and 3rd experiment, by 43% and 31 %,

respectively. It was assumed a decrease in the effectiveness of Rapamycin in the 1st experiment on REM cells, presumably due to something going wrong during storage of Rapamycin.


A
Wortmannin
ZSTK474
KP372-1
Rapamycin
Control
20 μM
Control
20 μM
Control
400 nM
Control
20 μM
Jurkat T
3132
SB
REM134
J3T
C2
Propidium Iodide (PI)
Annexin V


B
Wortmannin
ZSTK474
KP372-1
Rapamycin
Control
20 μM
Control
20 μM
Control
400 nM
Control
20 μM
Jurkat T
3132
SB
REM134
J3T
C2
Propidium Iodide (PI)
Annexin V
5% 36% 4% 21% 7% 64% 3% 64%
89% 21% 42% 91% 4% 64% 14% 81% 12% 5% 29% 94% 2% 5% 29%
3% 8% 1% 2% 1% 47% 1% 7%
94% 3% 77% 15% 98% 0% 94% 4% 92% 7% 9% 43% 96% 3% 14% 77%
4% 5% 4% 12% 15% 2% 44%
87% 7% 80% 12% 87% 7% 51% 19% 66% 16% 29% 22% 91% 6% 4% 51%
7% 7% 8% 7% 3% 89% 13% 19%
86% 6% 83% 9% 83% 9% 79% 13% 95% 2% 1% 9% 85% 1% 42% 38%
2% 2% 2% 2% 1% 97% 2% 1%
97% 0% 96% 1% 97% 1% 97% 1% 97% 2% 1% 2% 96% 1% 97% 2%
4% 6% 4% 9% 3% 73% 4% 15%
91% 5% 63% 31% 93% 4% 68% 22% 93% 4% 1% 23% 92% 4% 2% 83%

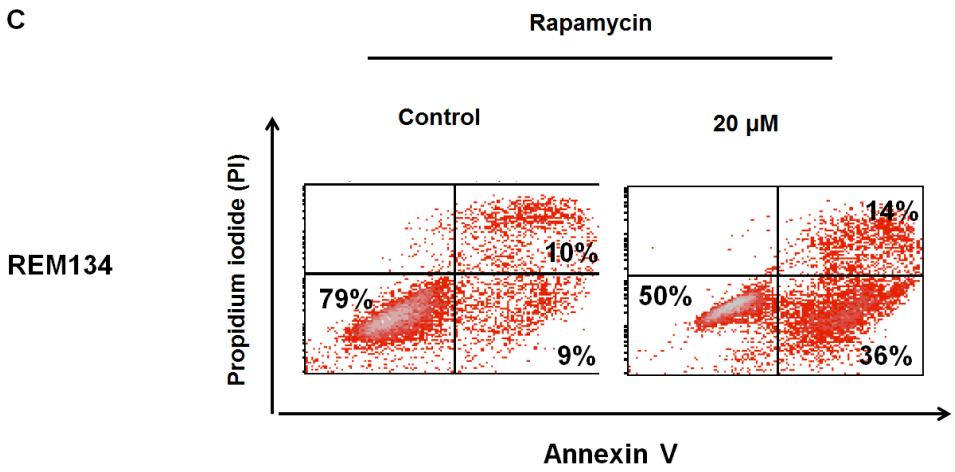


Figure 3.07. Effects of the class I PI3K pathway inhibitors on induction of apoptosis. Cells were treated with 20 μM Wortmannin for 2 dyas, 20 μM ZSTK474 for 2 days, 400 nM KP372-1 for 1 day, 20 μM Rapamycin for 2 days, or vehicle control. Induction of apoptosis was determined by annexin V/PI staining and analysis by flow cytometry (see Chapter 2, Section 2.5). Percentages of live, early apoptotic and late apoptotic cells were indicated in lower left, lower right and upper right quadrants, respectively, of bivariant dotplots. (A) and (B) represented two independent experiments (C) The 3rd experiment for REM134 cells incubated with/without Rapamycin.

3.3.2.4.3 Responses of REM, J3T and C2 cells to short exposure to KP372-1

As KP372-1 down-regulated phosphorylation of PI3K substrates in REM134 and J3T cells after 5 hour incubation (Figure 3.03C), annexin V/propidium iodide staining (see Chapter 2, Section 2.5) was performed to determine the relationship between PI3K pathway inhibition and the level of apoptosis. As shown in Figure 3.08, KP372-1 increased apoptosis of REM134 cells by 84%, followed by - in decreasing order of drug potency - J3T (65%) and C2 (31%).

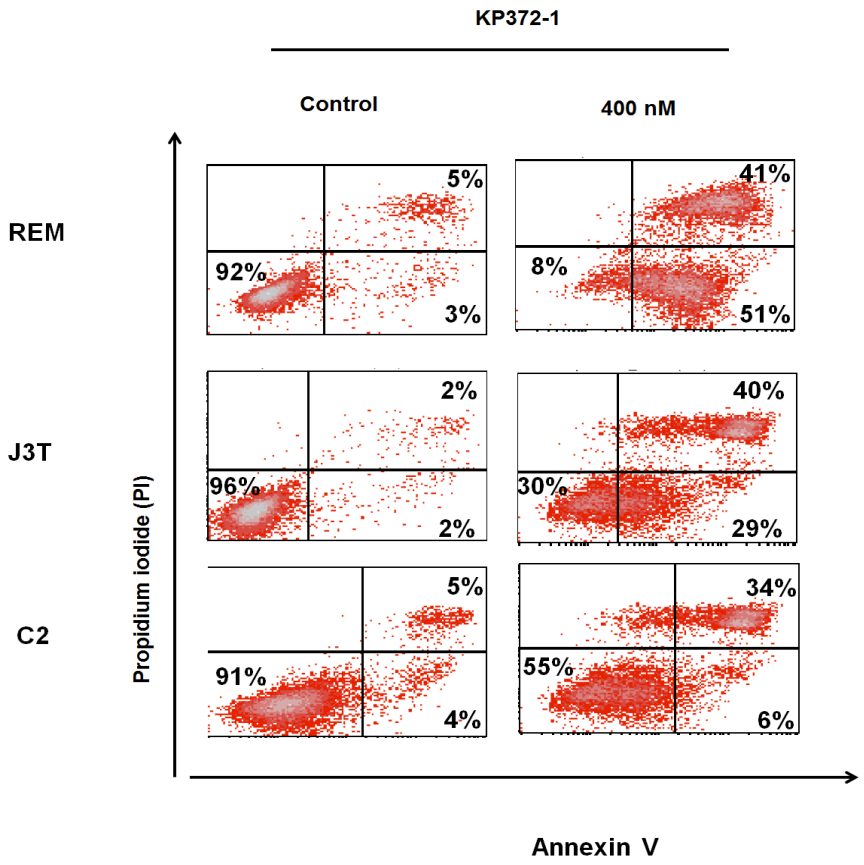


Figure 3.08. Significant increased apoptosis in REM134 and J3T cells after treatment of KP372-1 for 5 hrs. Cells were treated with 400 nM KP372-1 or vehicle control for 5 hours. Induction of apoptosis was determined by annexin V/PI staining and flow cytometry analysis (see Chapter 2, Section 2.5). Percentages of live, early apoptotic and late apoptotic cells were located at lower left, lower right and upper right quadrants, respectively.

3.3.3 Effects of Rapamycin combined with either Wortmannin or ZSTK474

To investigate the effects of simultaneous inhibition of both PI3K and mTOR signaling, cells were treated with Rapamycin combined with either Wortmannin or ZSTK474. The inhibitory effect of drug combination on cell viability was evaluated by using the Bliss additivism model (the methods used being described in Chapter 2, Section 2.4 and Section 2.7) (Buck *et al.* 2006). Briefly, if the cell viability rates generated by Bliss additivism model analysis are greater than, overlap, or are less than rates obtained from experimental results, a drug combination is interpreted as having synergistic, additive, or antagonistic effects, respectively.

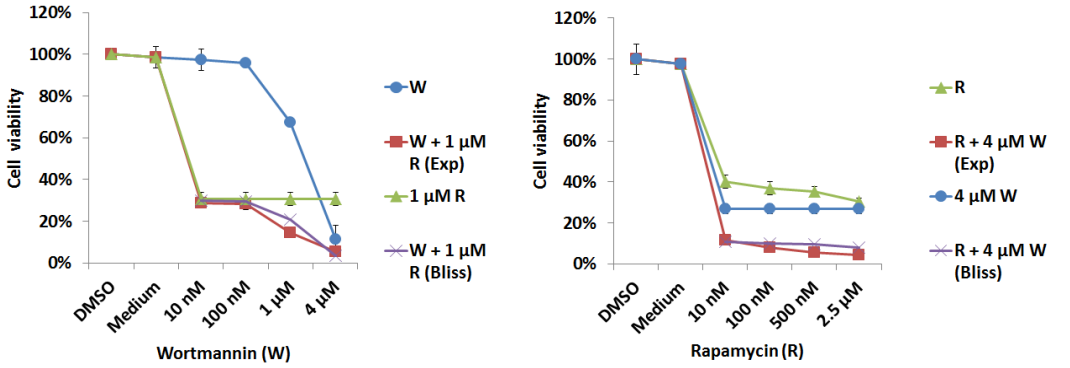
3.3.3.1 Rapamycin combined with Wortmannin showed variable inhibitory effects on cell viability

As shown in Figure 3.09 and Appendix 1, the Bliss analyses showed that simultaneous treatment with Wortmannin and Rapamycin inhibited the viability of Jurkat T, 3132 and J3T cells in an additive manner. Over the range of concentrations tested in this study, the drug combination increased efficacy in Jurkat T cells by 17-25% ($p<0.05$), 3132 by 7-18% ($p<0.05$), J3T cells by 6-8% ($p<0.05$), as compared with either Rapamycin or Wortmannin alone, depending on which single agent achieved maximal inhibition of cell viability.

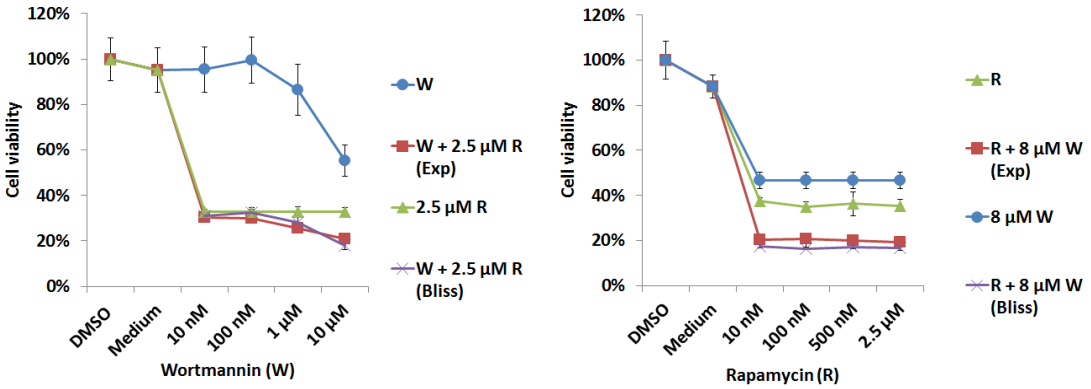
Wortmannin synergized with Rapamycin and in SB and C2 cells, increasing drug efficacy by 7-41% ($p<0.05$) and 23-41% ($p<0.05$), respectively.

However, the drug combination showed antagonistic effects on REM134 cells.

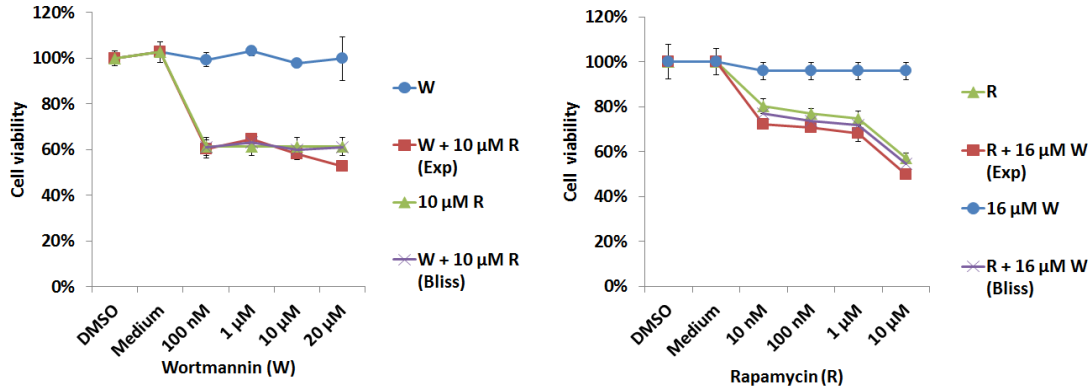
1. Jurkat T



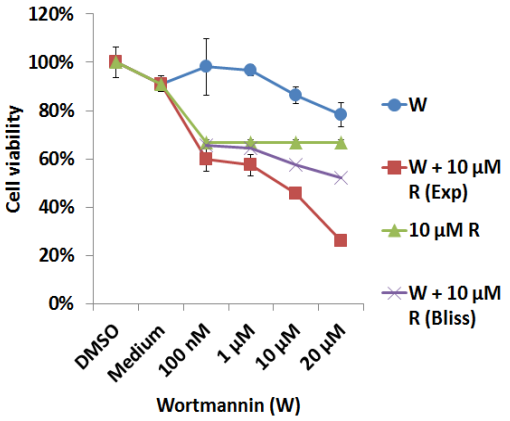
2. 3132

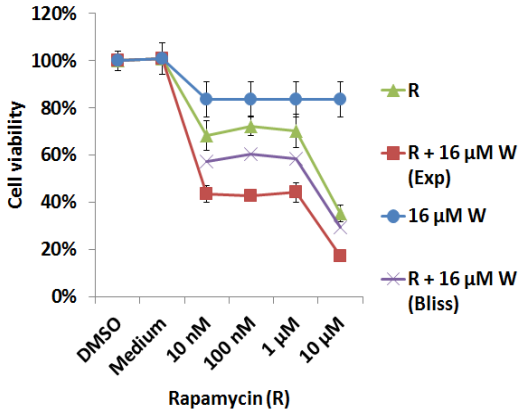


3. J3T

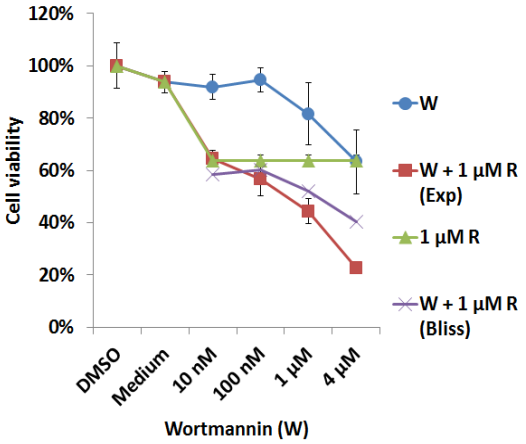


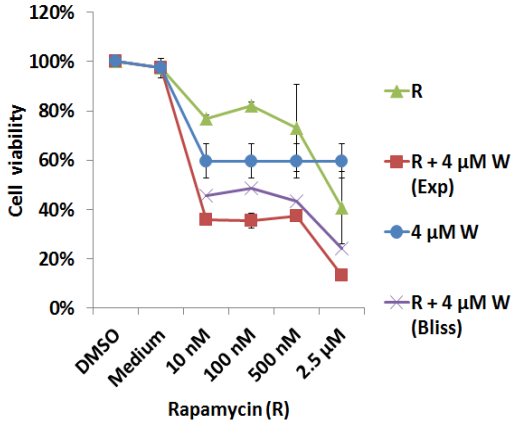
4. SB



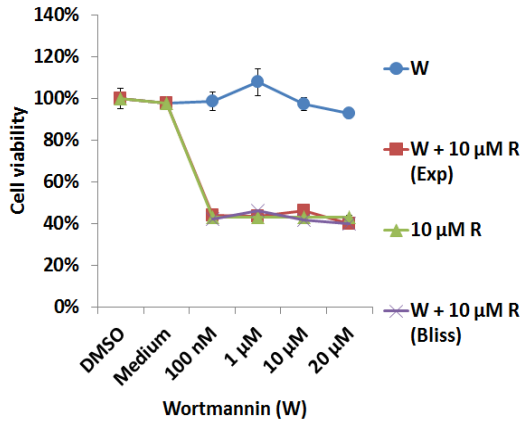


5. C2





6. REM134



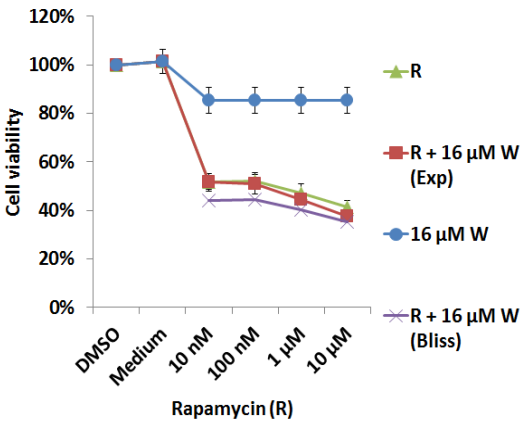


Figure 3.09. Cell viability in response to combined Rapamycin/Wortmannin treatment. Cells were treated with the indicated doses of Wortmannin, Rapamycin, and the combination of the former two inhibitors or vehicle control for 3 days. After drug treatment, the number of viable cells was determined by using CellTiter-Glo® Luminescent Cell Viability Assay (see Chapter 2, Section 2.4). Viability of the drug-treated cells was compared with the vehicle (DMSO)-treated cells on the same culture plates. Results were expressed as mean (±SD) counts of quadruplicate wells. Theoretical values for the combined inhibitory effects of the two drugs were obtained using the Bliss additivism model as described in Chapter 2, section 2.7. Statistical analysis to determine whether there is significant difference between two treatment groups was described in Chapter 2, Section 2.8 (p-value < 0.05 indicates significant difference). Bliss, Bliss theoretical value; Exp, Experiment value; R, Rapamycin; W, Wortmannin.

3.3.3.2 Rapamycin combined with ZSTK474 inhibited cell viability in an additive manner

As shown in Figure 3.10B and Appendix 2, the Bliss analyses showed that ZSTK474 combined with Rapamycin had an additive effect on most cell lines. However, statistical analyses indicated that the additive effects on REM134 were Insignificant. Over the range of concentrations tested in this study, the drug combination increased efficay in Jurkat T cells by 8-22% ($p<0.05$), 3132 by 16-23% ($p<0.05$), SB by 7-18% ($p<0.05$), REM134 cells by 9% ($p<0.05$), and C2 cells by 13-29% ($p<0.05$), as compared with either Rapamycin or ZSTK474 alone, depending on which single agent achieved maximal inhibition of cell viability.

ZSTK474 synergized with Rapamycin in J3T cells, increasing efficacy by 23-36% ($p<0.05$) when compared with a single agent.

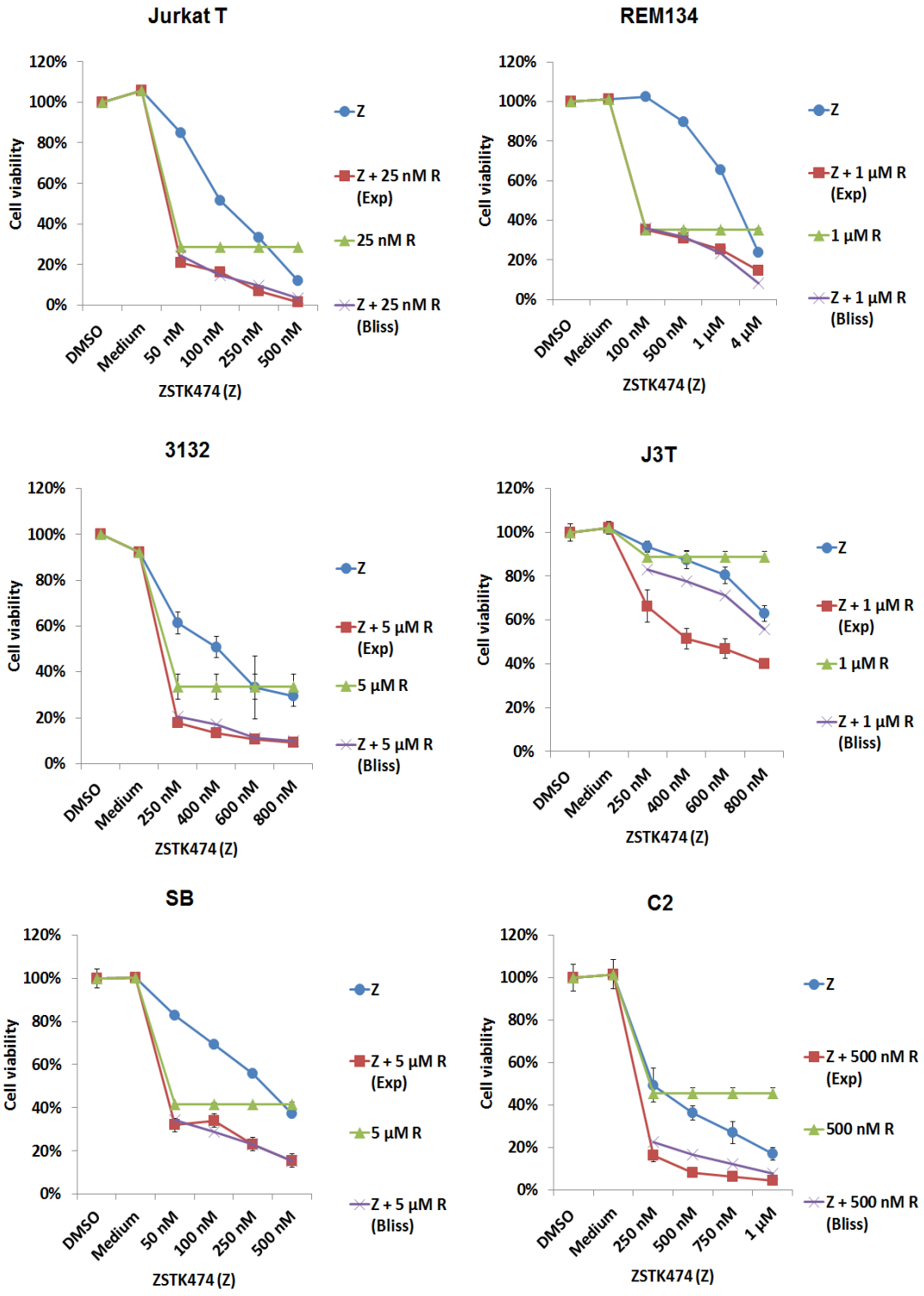


Figure 3.10. Rapamycin combined with ZSTK474 inhibited cell viability in

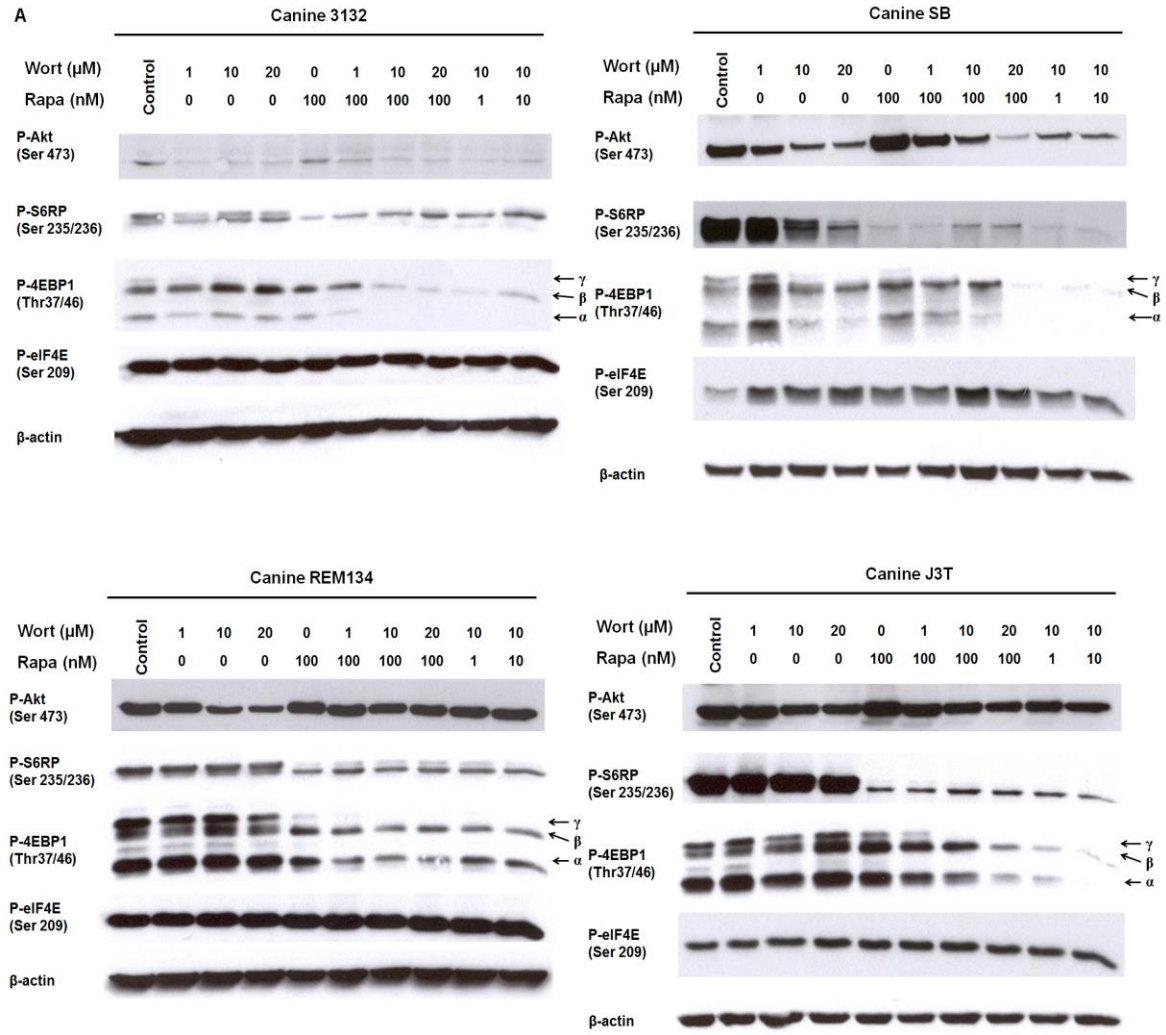
an additive or synergistic manner. Cells were treated with the indicated doses of Wortmannin, Rapamycin, and the combination of the former two inhibitors or vehicle control for 3 days. After drug treatment, the number of viable cells was determined by using CellTiter-Glo® Luminescent Cell Viability Assay (as described in Chapter 2, Section 2.4). Results were expressed as mean (±SD) counts of quadruplicate wells. Viability of the drug-treated cells was compared with the vehicle (DMSO)-treated cells on the same culture plates. Theoretical values for the combined inhibitory effects of the two drugs were obtained using the Bliss additivism model as described in Chapter 2, section 2.7. Statistical analysis to determine whether there is significant difference between two treatment groups was described in Chapter 2, Section 2.8 (p-value < 0.05 indicates significant difference). Bliss, Bliss theoretical value; Exp, Experiment value; R, Rapamycin; Z, ZSTK474.

3.3.3.3 Western blotting analysis on effects of Rapamycin combined with Wortmannin on the class I PI3K/Akt/mTOR signaling pathway

Western blot analysis was performed to dissect further the effects of combining Wortmannin with Rapamycin. As shown in Figure 3.11A, Western blot analysis revealed that the drug combination profoundly or fully inhibited 4E-BP1 phosphorylation in 3132, SB and J3T cells, but not in REM134 cells, at the time point of 5 hours. Prolonged exposure (for 9 hours) to the drug combination was observed to profoundly inhibit 4E-BP1 phosphorylation in all cell lines (Figure 3.11B). However, it appeared that there was no profound inhibition of S6RP phosphorylation, based on the same levels observed in the Rapamycin treatment group and the drug combination group in 3132, REM134 and J3T cells (Figure 3.11A).

It was observed that full suppression of 4E-BP1 phosphorylation did not contribute to down-regulation of eIF4E phosphorylation. In all cell lines except Jurkat T and SB cells, the phosphorylation levels were not altered by drug treatment. In Jurkat T

cells, eIF4E phosphorylation was up-regulated in response to Rapamycin alone, but this phenomenon was not observed in cells treated concomitantly with Rapamycin and Wortmannin. In SB cells, eIF4E phosphorylation was up-regulated in response to Rapamycin alone, Wortmannin alone and the combination of the both drugs. Besides, Akt phosphorylation was slightly up-regulated in SB cells treated with Rapamycin alone (Figure 3.11A and 3.11B).



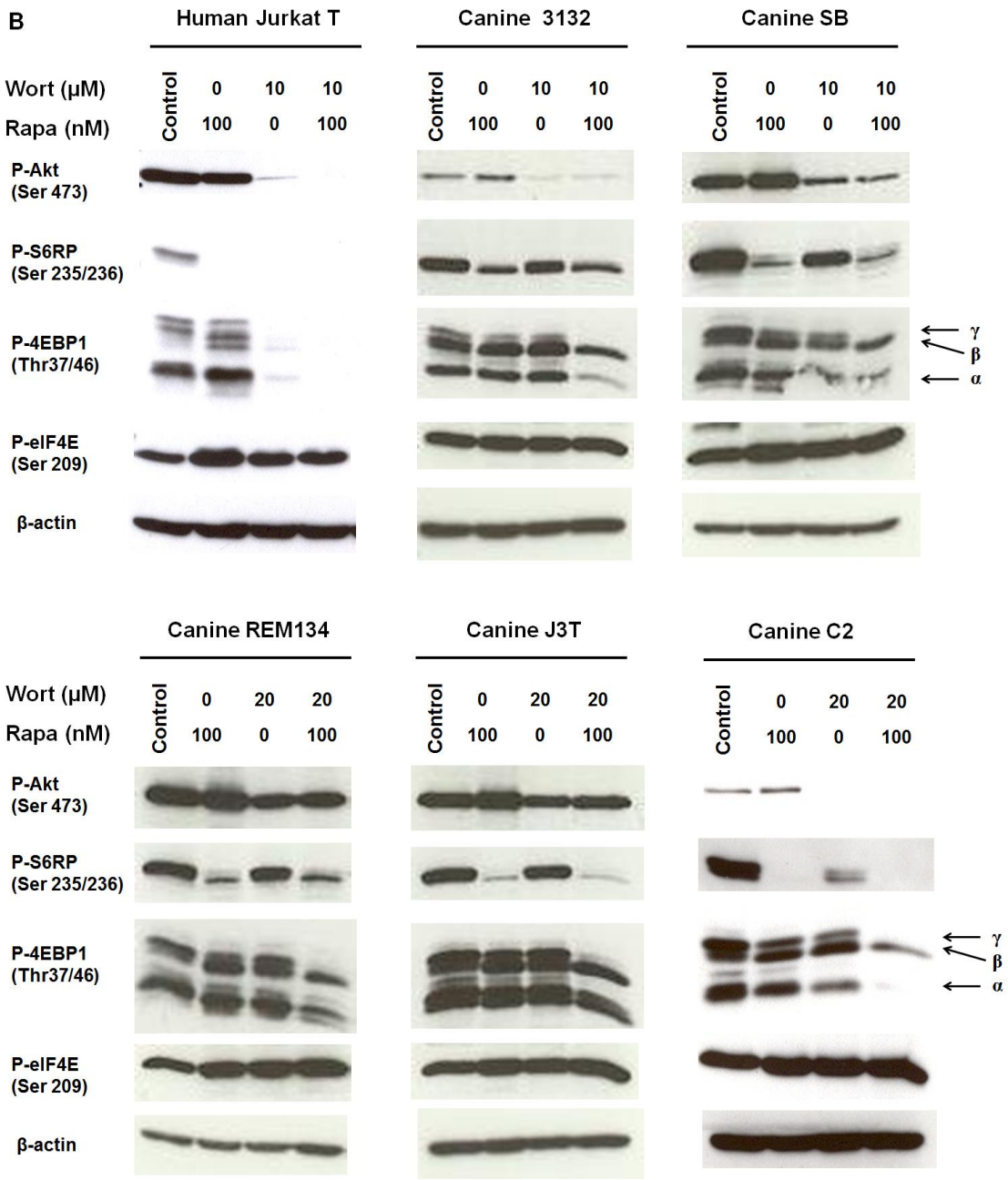


Figure 3.11. Western blot analysis on inhibitory effects of Rapamycin combined with Wortmannin on class I PI3K/Akt/mTOR axis pathway. Cells were seeded at a density of 20,000 cells/ml and incubated overnight, followed by treated with the indicated concentrations of Rapamycin, Wortmannin, or the

combination of Rapamycin and Wortmannin for 5 hours (A) or 9 hours (B). Whole cell lysates (comprising 50 μg total protein) were subjected to Western blotting analysis (described in Chpater 2, Section 2.6) with β-actin as a loading control. Three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α), were observed (Gingras *et al.* 1998).

3.3.3.4 Western blotting analysis on effects of Rapamycin combined with ZSTK474 on the PI3K/Akt/mTOR signaling pathway

The effect of treatment with a combination of Rapamycin and the pan-class I PI3K inhibitor ZSTK474 was also evaluated using Western Blot analysis. The effect of this drug combination was similar to that seen with combined Rapamycin/Wortmannin treatment. As shown in Figure 3.12, the drug combination profoundly inhibited 4E-BP1 phosphorylation only. Again, the full inhibition of 4E-BP1 phosphorylation did not further down-regulate eIF4E phosphorylation. For 3132, SB, REM134 and J3T cells treated with both drugs, inhibition of S6RP phosphorylation equivalent to that produced by Rapamycin alone. For Jurkat T cells, whilst Rapamycin alone up-regulated eIF4E phosphorylation, this was not observed in response to combined Rapamycin/ZSTK474. Akt phosphorylation levels in Jurkat T cells were decreased by Rapamycin after incubation for a longer time period (18 hours).

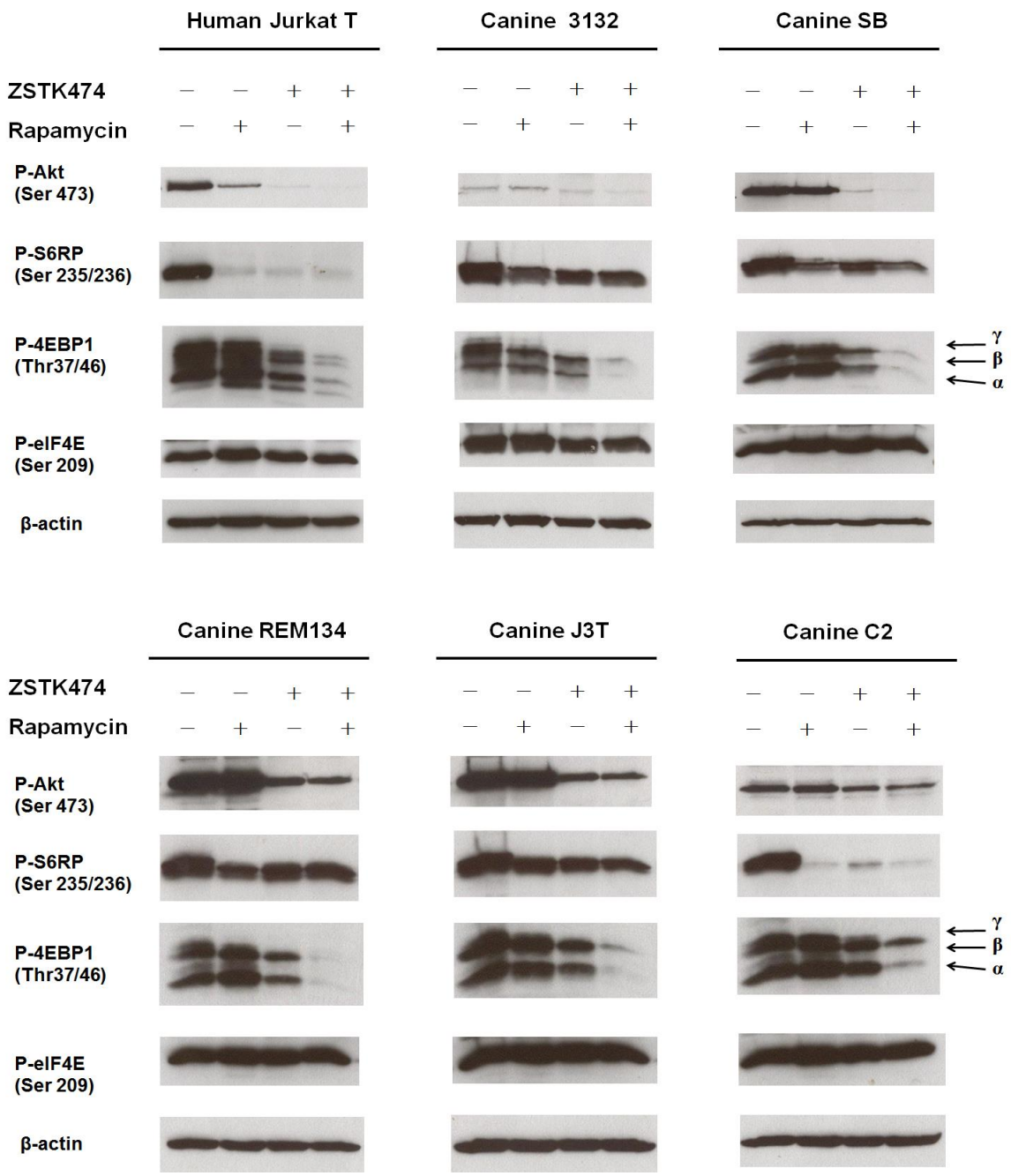


Figure 3.12. Western blot analysis on inhibitory effects of Rapamycin combined with ZSTK474 on class I PI3K/Akt/mTOR axis pathway. Cells were

seeded at a density of 20,000 cells/ml and incubated overnight, followed by treated for 18 hours with 5 μM* ZSTK474, 100 nM Rapamycin, or a combination of both inhibitors. Whole cell lysates (comprising 50 μg total protein) were subjected to Western blotting analysis (as described in Chapter 2, Section 2.6) with β-actin as a loading control. *(10 μM ZSTK474 for REM134 cells). Three 4EBP1 isoforms, including a hyperphosphorylated isoform (γ), a middle form (β), and an unphosphorylated isoform (α), were observed (Gingras *et al.* 1998).

3.3.4 Effects of the combination of the class I PI3K inhibitors and Doxorubicin on cell viability

To investigate whether the PI3K/Akt/mTOR signaling affects the sensitivity to conventional chemotherapy, the effects of combined treatment with PI3K pathway inhibitors and Doxorubicin were evaluated for SB, REM134, 3132 and C2 cells and analyzed using Bliss additivism model (as described in Chapter 2, Section 2.7).

As shown in Figure 3.13A and Appendix 3, the Bliss analysis predicted that the combination of Doxorubicin and PI3K pathway inhibitors decreased the viability of SB cells in an additive or synergistic manner. Wortmannin, ZSTK474 and Rapamycin combined with Doxorubicin showed additive effects, which increased drug efficacy by 5-16% ($p<0.05$), 4-13% ($p<0.05$) and 5-23% ($p<0.05$), respectively, as compared with a single agent, depending on which single agent achieved maximal inhibition of cell viability.

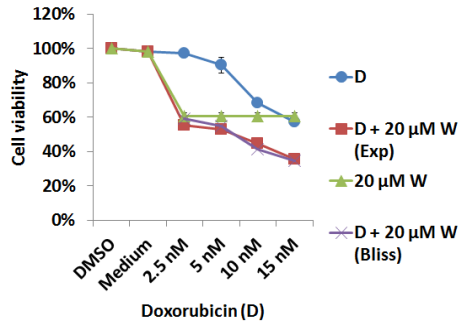
The Bliss analysis predicted that KP372-1 combined with Doxorubicin had a synergistic effect on SB cells (Figure 3.13A). However, the statistical analyses only confirmed the synergism in one dataset, which was obtained from 80 nMKP372-1 group versus 80 nM KP372-1 combined with 2.5 nM Doxorubicin group. In this dataset, the combination of KP372-1 and 2.5 nM Doxorubicin increased drug efficacy by 13% ($p<0.05$), as compared with KP372-1 alone (Figure 3.13A and Appendix 3).

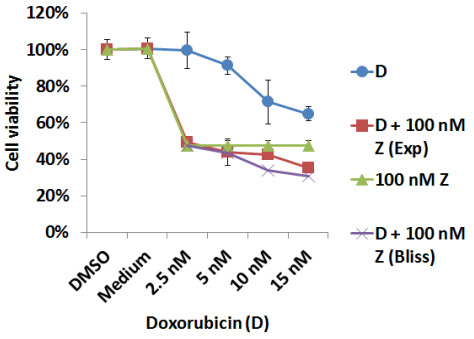
With regard to REM cells, the Bliss analysis showed that Wortmannin, ZSTK474 and KP372-1 antagonized the inhibitory activity of Doxorubicin towards REM134 cells. Only Rapamycin combined with Doxorubicin produce a complementary effect (additive) with an increase in efficacy of 13% ($p<0.05$), as compared with Rapamycin alone (Figure 3.13B and Appendix 3).

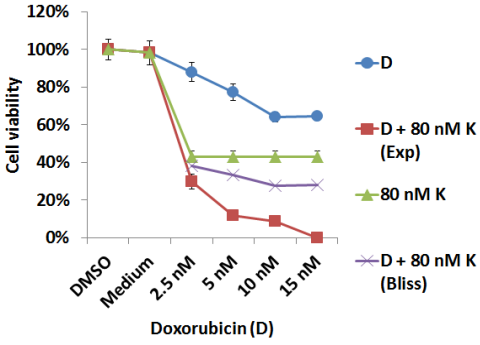
For 3132 and C2 cells, Wortmannin, ZSTK474, and Rapamycin combined with Doxorubicin inhibited viability in an antagonistic manner (Figure 3.13C and 3.13D).

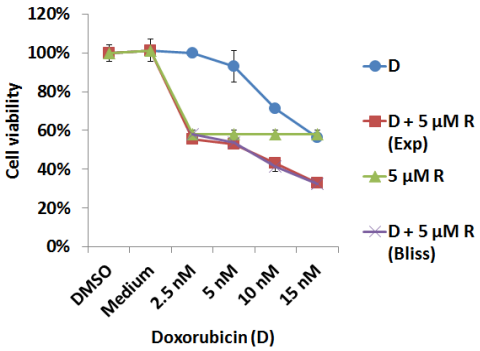
A

SB


120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 20 µM W (Exp)
20 µM W
D + 20 µM W (Bliss)

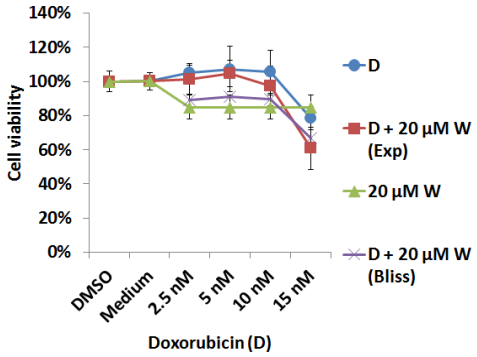

120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 100 nM Z (Exp)
100 nM Z
D + 100 nM Z (Bliss)

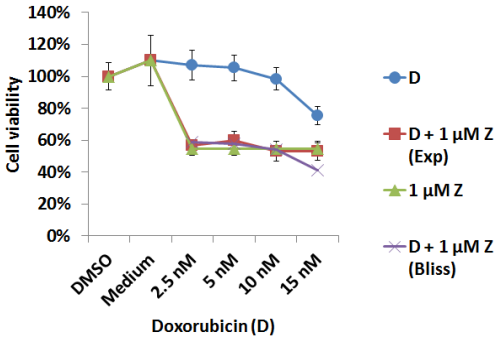

120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 80 nM K (Exp)
80 nM K
D + 80 nM K (Bliss)

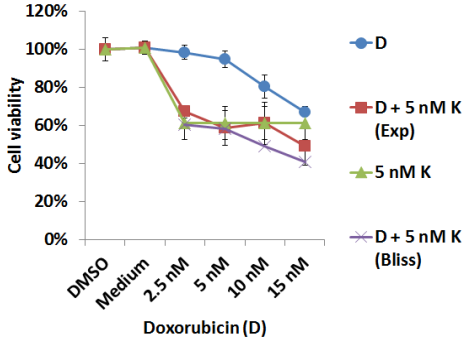

120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 5 µM R (Exp)
5 µM R
D + 5 µM R (Bliss)

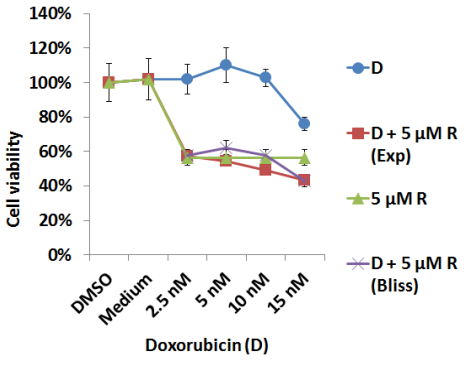
B

REM134


140%
120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 20 µM W (Exp)
20 µM W
D + 20 µM W (Bliss)


140%
120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 1 µM Z (Exp)
1 µM Z
D + 1 µM Z (Bliss)


120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 5 nM K (Exp)
5 nM K
D + 5 nM K (Bliss)


140%
120%
100%
80%
60%
40%
20%
0%
Cell viability
DMSO
Medium
2.5 nM
5 nM
10 nM
15 nM
Doxorubicin (D)
D
D + 5 µM R (Exp)
5 µM R
D + 5 µM R (Bliss)

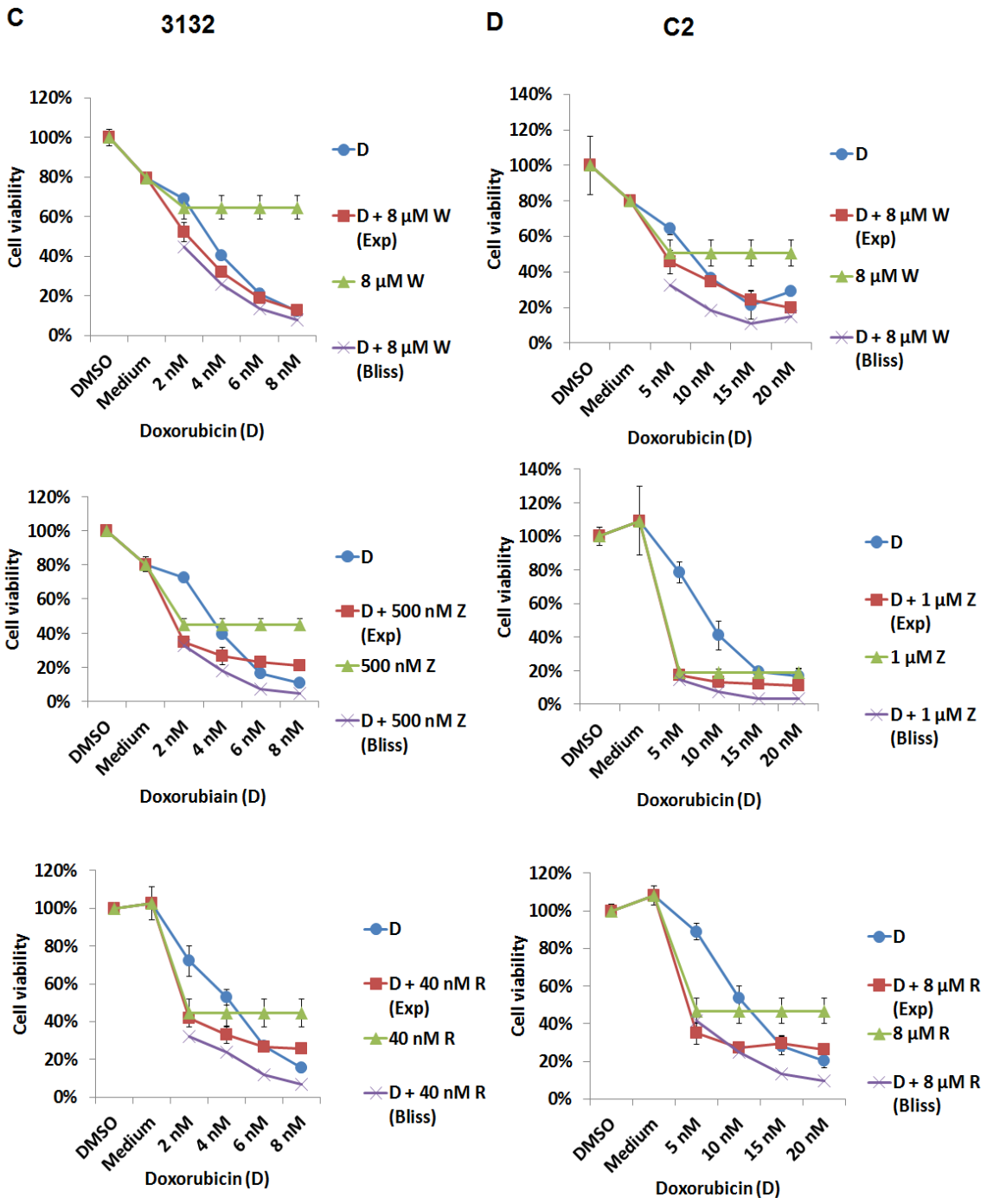


Figure 3.13. Effects of the combination of the PI3K/Akt/mTOR pathway inhibitors and Doxorubicin on cell viability. Canine SB (A), REM134 (B), 3132

(C), and C2 (D) cells were treated with the indicated does of the PI3K/Akt/mTOR pathway inhibitors, Doxorubicin, a combination of these drugs, or vehicle control for 3 days (except KP372-1 for 2 days). After drug treatment, the number of viable cells was determined by using CellTiter-Glo® Luminescent Cell Viability Assay (as described in Chapter 2, Section 2.4). Results were expressed as mean (±SD) counts of quadruplicate wells. Viability of the drug-treated cells was compared with the vehicle (DMSO)-treated cells on the same culture plates. Theoretical values for the combined inhibitory effects of the two drugs were obtained using the Bliss additivism model as described in Chapter 2, section 2.7. Statistical analysis to determine whether there is significant difference between two treatment groups was described in Chapter 2, Section 2.8 (p-value < 0.05 indicates significant difference). Bliss, Bliss theoretical value; D, Doxorubicin; Exp, Experiment value; R, Rapamycin; W, Wortmannin; Z, ZSTK474.

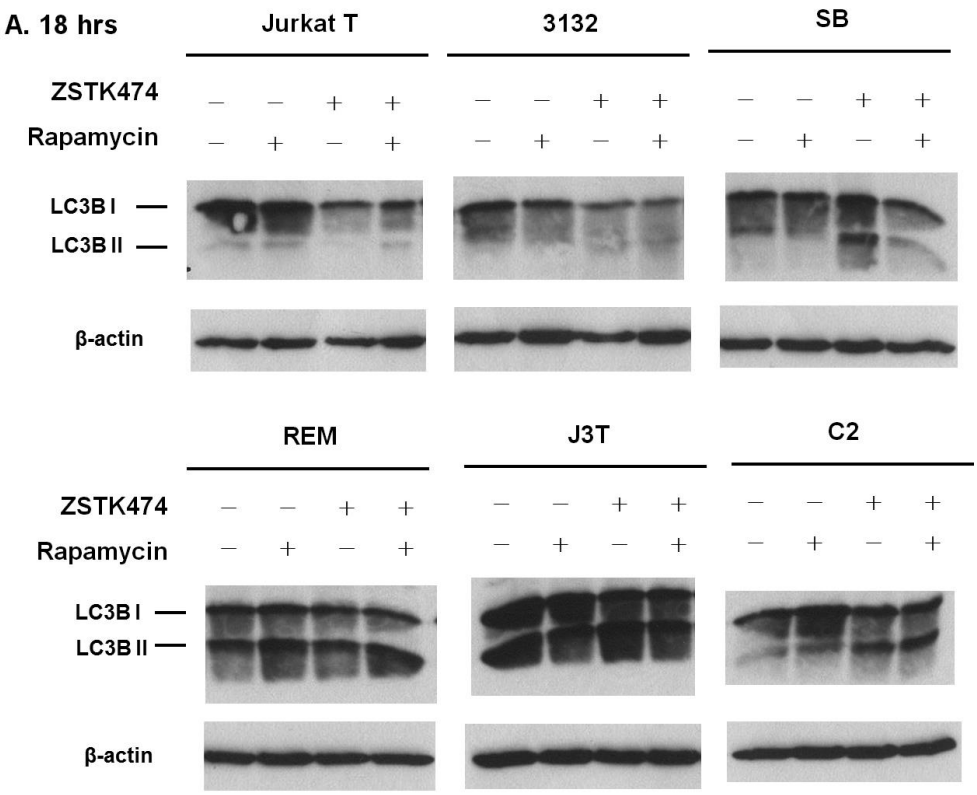
3.3.5 Effects of the class I PI3K pathway inhibitors on autophagy induction

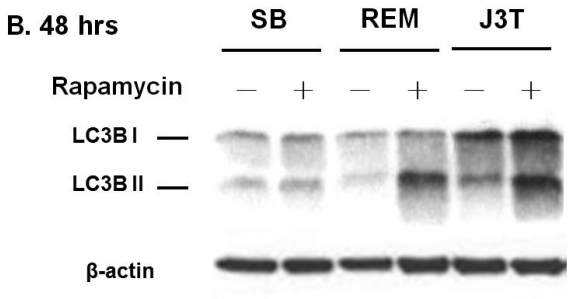
Accumulating evidence suggests that mTORC1 signaling is a negative regulator of autophagy that may protect tumour cells from apoptosis under poor conditions such as stress and nutrient deprivation. Cells were treated with low doses of PI3K pathway inhibitors, followed by Western blot analysis on expression amounts of isoform B of human microtubule-associated protein 1 light chain 3 (LC3B) (Aoki *et al.* 2008). During autophagy, LC3B is converted from cytosolic LC3B-I to autophagosome membrane-bound LC3B-II, allowing LC3B-II to serve as an indicator of autophagy induction.

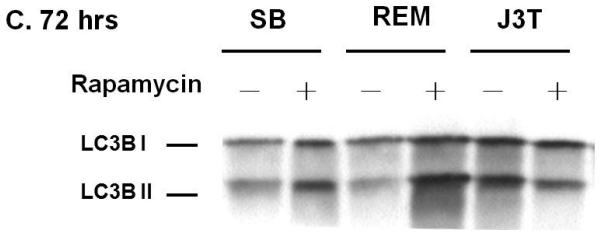
As shown in Figure 3.14A, REM134, J3T and C2 cells exposed to Rapamycin for 18 hours expressed similar levels of LC3B-II to their controls whereas Jurkat T, 3132 and SB cells did not express LC3B-II in response to Rapamycin. Prolonged treatment (48-72 hours) of Rapamycin was performed on SB, REM134, J3T and 3132 cells (Figure 3.14B and 3.14C). It was observed that a slight increase in LC3B-II level was observed in SB cells following 72 hour post-drug treatment. Drug-treated REM134 cells

expressed higher levels of LC3B-II through 48-72 hours as compared with the controls. The data obtained from J3T cells were paradoxical. Whilest J3T cells exposed to Rapamycin for 48 hours expressed higher LC3B-II levels, 72 hour post-drug treatment of J3T cells was observed to have less amount of LC3B-II as compared with the control (Figure 3.14B and 3.14C). For 3132 cells, expression of LC3B-II was not observed in the Rapamycin-treated cells, except the cells with 5 μM Rapamycin treatment for 3 hours (Figure 3.14D).

With regard to ZSTK474 treatment, increased levels of LC3B-II expression were observed in ZSTK474-treated SB and C2 cells, as compared with the controls. When combined ZSTK474 and Rapamycin, induction of autophagy was only observed in SB cells (Figure 3.14A).


A. 18 hrs
Jurkat T
3132
SB
ZSTK474
Rapamycin
LC3B I
LC3B II
β-actin
REM
J3T
C2
ZSTK474
Rapamycin
LC3B I
LC3B II
β-actin


B. 48 hrs
SB
REM
J3T
Rapamycin
LC3B I
LC3B II
β-actin


C. 72 hrs
SB
REM
J3T
Rapamycin
LC3B I
LC3B II

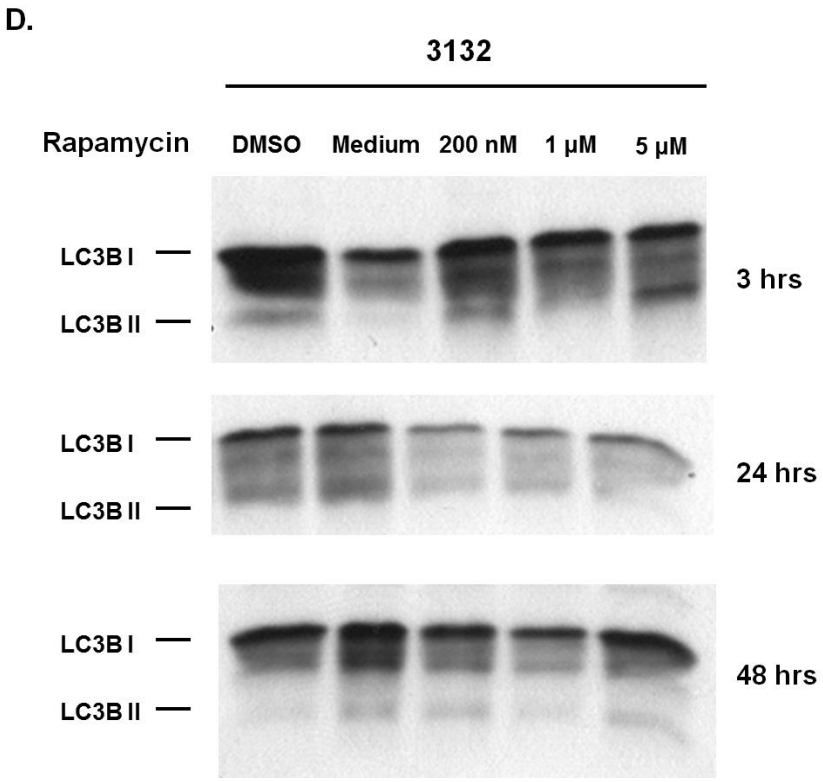


Figure 3.14. Evaluation of the effects of Rapamycin and ZSTK474 on autophagy induction. (A) Cells were treated for 18 hours with 5 µM ZSTK474 (10 µM ZSTK474 for REM134 cells), 100 nM Rapamycin, a combination of both inhibitors or vehicle control. (B) Cells were treated with 200 nM Rapamycin for 48 hours. (C) Canine 3132 cells were treated with Rapamycin at the indicated concentrations for 3, 24, or 48 hours. Whole cell lysates (comprising 50 µg total protein) were subjected to Western blot (described in Chapter 2, Section 2.6) with the indicated antibodies. β-actin was used as a loading control.

3.4 Discussion

3.4.1 Active class I PI3K/Akt/mTOR signaling in canine cancer cells

Accumulating evidence has demonstrated the critical role of class I PI3K/Akt/mTOR signaling in tumourigenicity in a wide array of human cancer types through a series of preclinical studies of either stimulation or shut-down of this pathway (Altomare and Testa 2005; Chiang and Abraham 2007; Engelman 2009). Moreover, some inhibitors that specifically target this pathway have been approved for the treatment of renal and pancreatic cancers in clinic (Kwitkowski *et al.* 2010; Saif 2011). So far, only few reports concerning the role of this pathway in canine oncology have been published (Yuan *et al.* 2009; Kwitkowski *et al.* 2010; Wang 2010). In the present study, five cell lines derived from a variety of canine tumours were selected for investigation into the impact of this pathway on canine tumourigeneity. Canine REM134 and human Jurkat T cells were demonstrated to constitutively express this signaling, as evidenced by loss of PTEN and expression of phosphorylated Akt. Evidence of active class I PI3K/Akt/mTOR axis signaling was also demonstrated for the other tested canine cell lines, with detectable levels of phosphorylated forms of PI3K downstream effectors, including Akt, mTOR, S6RP, 4EBP1 and eIF4E.

Although Akt was activated in all lines, 3132 cells were found to express much lower levels of phosphorylated Akt than the other cell lines. Previous studies of mouse embryonic fibroblasts (MEFs) suggested a positive correlation of knockdown of TSC1 or TSC2 gene expression with decreased phosphorylation of Akt on Ser473 (Zhang *et al.* 2003; Harrington *et al.* 2004; Shah *et al.* 2004). More recently, it has been reported that the TSC1-TSC2 complex interacts with mTORC2 to positively regulate mTORC2-mediated Akt phosphorylation, whereas the complex acts as a negative regulator of mTORC1 signaling (Huang *et al.* 2008). As the Western blot profile (Figure 3.01) shows that 3132 cells exhibit active mTORC1 signaling and minimal Akt phosphorylation, it is possible that molecular alterations of the TSC1-TSC2 complex occur in 3132 cells.

Indeed, LOH and somatic mutations in *TSC1* or *TSC2* are described in many types of cancer (Niida *et al.* 2001). Using polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) analysis of DNA or complementary DNA (cDNA) of *TSC1* and *TSC2* genes in canine 3132 cells or Western blot analysis of expression status of TSC1-TSC2 complex can help to clarify whether dysfunction of TSC1-TSC2 complex contributes to low levels of Akt phosphorylation in this cell line (Hayashi 1991; Henske *et al.* 1996). An alternative explanation for the low levels of Akt phosphorylation in 3132 cells would be due to low expression level of total Akt kinase. Further investigation of expression levels of total Akt by using Western blotting may provide a clearer understanding of Akt activity in 3132 cells.

Levels of Akt phosphorylation for canine J3T, SB and C2 cells were comparable to those of PTEN-null REM134 and and Jurkat T cells. High levels of phosphorylated Akt might presumably result from any molecular alterations of Akt upstream regulators, such as RTKs, Ras, and class I PI3Ks. Indeed, a recent study of cell lines derived from a variety of human cancer types has revealed that activating mutations of *Ras* alone or coupled with mutations of *PIK3CA* leads to PTEN-positive cells exhibiting high levels of phosphorylated Akt (Ihle *et al.* 2009). In addition, hyperactivity of mTORC2 as a result of over-expression of the Rictor subunit can contribute to up-regulation of Akt phosphorylation in some glioma cell lines (Masri *et al.* 2007). Further investigation of somatic mutations (including deletion, insertion and single nucleotide substitution) of the genes encoding upstream regulators of Akt in all cell lines could be carried out by using PCR amplification and DNA sequencing analysis, followed by using computer softwares such as mutation detection algorithms and single nucleotide polymorphisms (SNPs) detector to analyze and identify genetic mutations (Ding *et al.* 2008). In addition, dysregulation of genes encoding RTKs, such as KIT, VEGFR and EGFR, and class I PI3Ks such as p110α and p110β catalytic subunits, is well-documented in cancers, and analysis of copy number gains of these genes using quantitative PCR may help to clarify the mechanism for constitutive activation of Akt (Humphrey *et al.* 1988; Knobbe and Reifenberger 2003; Liu *et al.* 2008). Semi-quantitative RT-PCR evaluating mRNA

expression levels of oncogenic RTK, class I PI3K and PDK1 might identify overexpression of oncoproteins resulting from elevated mRNA levels in the absence of gene amplification. As phosphorylation of multiple serine/threonine residues at C-terminus of PTEN may result in down-regulation of activity, Western blot analysis to determine the phosphorylation status at these sites (Ser380; Thr382/383) could be performed (Vazquez *et al.* 2001; Birle *et al.* 2002; Dal Col *et al.* 2008; Silva *et al.* 2008).

As active class I PI3K/Akt/mTOR signaling was detected in all tested cell lines, the class I PI3K pathway was subsequently inhibited at different levels by using the small molecules inhibitors Wortmannin, ZSTK474, KP372-1 and Rapamycin. The former two inhibitors specifically target all p110 isoforms of class IA and IB PI3Ks, whereas KP372-1 and Rapamycin specifically inhibit Akt and mTOR, respectively (Nakanishi *et al.* 1995; Huang *et al.* 2003; Mandal *et al.* 2005; Kong and Yamori 2007).

3.4.2 pan-class I PI3K inhibitors

3.4.2.1 Wortmannin and ZSTK474

The current results show that ZSTK474, KP372-1 and Rapamycin significantly down-regulate cell viability whereas cells show variable responses to Wortmannin. Wortmannin and ZSTK474 are both pan-class I PI3K inhibitors, but additional factors may account for the contrasting effects of the two drugs on cell viability. Wortmannin acts as a non-ATP competitive inhibitor towards class I PI3K through the irreversible interaction between Lys802 residue of p110α and the furan ring (at C-20) of Wortmannin, thus preventing p110α from transferring the γ-phosphate group from ATP to PtdIns(4,5)P_2 (Wymann *et al.* 1996). By contrast, ZSTK474 competes with ATP for the ATP-binding pocket in the p110 catalytic subunit of class I PI3K, resulting in arrest of PtdIns (3,4,5)P_3 generation (Kong and Yamori 2007). Although Wortmannin acts as an irreversible pan-class I PI3K inhibitor, this drug is reported to have poor stability in cell medium, with a half life of 8-13 mins (Holleran *et al.* 2003). Moreover, the efficacy towards class I PI3K signaling, as measured by inhibition of Akt phosphorylation, only

lasts for ~18 hours (Howes *et al.* 2007). Consistent with previous findings, Wortmannin in this study displays shorter duration (~12 hours) of suppression of Akt phosphorylation than ZSTK474. Moreover, it has been demonstrated that ZSTK474 is a more potent inhibitor of class I PI3K/Akt/mTOR axis signaling than Wortmannin, as evidenced by decreased phosphorylation levels of not only Akt but also S6RP and 4EBP1 in ZSTK474-treated cells. Hence, it appears that viability of all cell lines tested in this study, is at least in part, class I PI3K-dependent.

The hypothesis of class I PI3K-driven cell viability in all lines is further supported by previous findings. Wortmannin is reported to target p110α, p110β, p110γ and p110δ catalytic subunits of class I PI3K with an inhibitory concentration for 50% kinase activity (IC-50) of 4 nM, 1 nM, 4 nM and 9 nM, respectively (Kong and Yamori 2008). Moreover, this inhibitor showed ≥ 100 fold selective inhibition for class I PI3Kα over other classes of PI3K such as class II PI3K-C2β and class III PI3K (yeast Vps34) (Nakanishi *et al.* 1995; Stein 2001). Several serine/threonine kinases that contain catalytic domains resembling PI3Ks such as PI4Ks, mTOR, ATM and DNA-PK as well as non-PI3K-related kinase such as myosin light chain kinase (MLCK) have been shown to have 20-50 fold IC50 values of Wortmannin as compared with p110α (Stein 2001).

In contrast to Wortmannin, evidence suggests that ZSTK474 is a more specific inhibitor of class I PI3K. ZSTK474 inhibits four p110 isoforms of class I PI3K with IC-50 values ranging from 5-49 nM (Kong and Yamori 2007). It has been demonstrated to have ~11, ~24, and ~27 fold specific inhibition for class I PI3Kα (IC-50 value of 16 nM) over class II PI3K-C2β, mTOR and DNA-PK, respectively. Moreover, ZSTK474 showed weak or no inhibitory effects on activities of class II PI3K-C2α, class III PI3K, and PI4K. In addition, ZSTK474 did not down-regulate phosphorylation of ERK and activities of several components of MAPK pathway (Yaguchi *et al.* 2006; Kong and Yamori 2007; Kong *et al.* 2009; Kong *et al.* 2010). Thus, the more restricted activity of ZSTK474 as compared with Wortmannin suggests that the viability of all lines is, in part, driven by active class I PI3K.

3.4.2.2 Inhibitory activity of ZSTK474 is not mediated through induction of apoptosis

Although both pan-class I PI3K inhibitors show similar flow cytometry profiles for annexin V/PI staining, with no significant increase in apoptosis for most canine lines, both Wortmannin and ZSTK474 induce apoptosis of Jurkat T and C2 cells, with the former showing the more potent effect. However, ZSTK474 effectively down-regulates the viability of most lines and fully inhibits the proliferation of Jurkat T and SB cells. This suggests that ZSTK474 does not inhibit cell viability entirely through induction of apoptosis. A recent study of human cancer cell lines showed that ZSTK474 induces cell cycle arrest through inhibition of phosphorylation or expression of Akt and/or mTORC1 substrates, such as phospho (p)-GSK3β, p-mTOR, p-p70S6K and cyclin D1. However, ability to induce apoptosis is cell line dependent and ZSTK474 is considered, in general, a weak inducer of apoptosis (Yaguchi *et al.* 2006). As Western blot analysis demonstrated that ZSTK474 inhibits class I PI3K/Akt/mTOR axis signaling, the current findings suggest that class I PI3K is critical to the viability of cancer cell lines but implicates the mechanism of ZSTK474 to be largely through inhibition of Akt/mTORC1-mediated protein synthesis and cell growth rather than apoptosis induction.

3.4.2.3 Wortmannin inhibits cell viability through effects on class I PI3K/Akt/mTOR signaling and induction of apoptosis

In this study, Wortmannin fully inhibited cell viability in Jurkat T and C2 cells and had moderately inhibitory effects on 3132 and SB cells, whereas no inhibition of REM134 and J3T cells was observed. Further dissection of the mechanism using Western blot revealed that Wortmannin significantly decreased Akt phosphorylation in Jurkat T, 3132, SB and C2 cells, and also decreased phosphorylation of mTOR substrates in Jurkat T and C2 cells. This suggests a positive correlation between activation of the class I PI3K/Akt/mTOR axis and cell viability.

Wortmannin significantly induced apoptosis of Jurkat T and C2 cells but had no pro-apoptotic effects on the other lines (see Figure 3.07A and B). It is suggested that cell inhibitory effect of Wortmannin to occur through not only inhibition of class I PI3K/Akt/mTOR axis signaling but also apoptosis induction. However, since specific inhibition by ZSTK474 of class I PI3K weakly promotes apoptosis (see Figure 3.07A and B), the possibility that Wortmannin decreases cell survival through targeting additional protein kinases cannot be ruled out.

3.4.2.4 ZSTK474 does not fully inhibit cell viability in most lines.

Canine 3132, J3T, REM134 and C2 cells are sensitive to the specific PI3K inhibitor ZSTK474, as demonstrated by cell viability assays showing ≥74% inhibition of proliferation at the concentration of 10 μM. However, ZSTK474 fails to fully inhibit cell viability in most of the tested canine cell lines, suggesting the existence of another mechanism for cell survival. The active Erk signaling detected in these canine cells may play a role in resistance to PI3K pathway inhibition because the functions of class I PI3K/Akt/mTOR pathway and Erk pathway are partially overlapped. Erk has been reported to regulate cellular responses, including proliferation, survival, cap-dependent translation (through its downstream Mnk/eIF4E pathway), nuclear response to stress, transcription, and immediate-early response (Roux and Blenis 2004). In addition, both class I PI3K/Akt/mTOR and Raf/Mek/Erk pathways are downstream of RTKs and Ras (Lemmon and Schlessinger 2010; Castellano and Downward 2011).

3.4.2.5 Maximum tolerated dose (MTD) for Wortmannin and ZSTK474

In addition to challenges posed by its pharmacological properties (instability, poor solubility), studies *in vivo* of both murine tumours and human tumour xenografts in murine hosts indicated that Wortmannin produced severe hepatic and hematological toxicities. Whilst this has prevented Wortmannin from being taken forward into clinical trials, the compound remains widely used in vitro for signaling studies (Schultz *et al.* 1995; Ihle *et al.* 2004).

Recently, new derivatives of Wortmannin, such as PX-866, have been developed to overcome these pharmacological drawbacks. PX-866 has been applied to *in vitro* and *in vivo* experiments; as yet, there is no data published from clinical trials (Howes *et al.* 2007; Koul *et al.* 2010). A pharmacokinetic study of PX-866 reports its maximum tolerated dose (MTD) in the mouse model is 19.5 mg/kg, much greater than Wortamnnin (3 mg/kg) (Ihle *et al.* 2004).

As ZSTK474 is a novel and orally administered drug, available data regarding MTD for ZSTK474 is currently confined to *in vivo* experiments (Anzai *et al.* 2011). Weight loss was the only side effect that was observed in the mouse models of human tumour xenografts or artheritis when administered orally at doses up to 800 mg/kg, suggesting that ZSTK474 was well-tolerated in the mouse species (Yaguchi *et al.* 2006; Toyama *et al.* 2010).

3.4.3 KP372-1

3.4.3.1 KP372-1 is a potent inducer of apoptosis

In this study, KP372-1 was observed to produce the most potent inhibition of cell viability, indicating the critical role for Akt in the tested cell lines. Western blot analysis demonstrated that, compared with ZSTK474 and Rapmycin, high doses of or prolonged exposures to KP372-1 are required to inhibit Akt/mTORC1 signaling. However, KP372-1 showed remarkable efficacy for inducing apoptosis. A previous study of KP372-1 on AML cells / cell lines / xenografts suggests that this drug predominantly acts through inhibition of the PDK1/Akt-mediated anti-apoptotic mechanism, but has no effect on cell cycle progression (Zeng *et al.* 2006). In agreement with this study, our data suggests that KP372-1 is a potent inducer of apoptosis through down-regulation of Akt-mediated survival signaling but has less effect on inhibition of Akt/mTORC1-mediated activities such as protein synthesis and cell cycle progression. In addition, as REM134 cells are highly sensitive to KP372-1 but relatively resistant to Rapamycin, it is suggested that

Akt-mediated anti-apoptosis activity (rather than mTORC1 activity) is critical for the viability of REM134 cells.

3.4.3.2 Decreased phosphorylation of Akt and eIF4E is presumably due to apoptotic effect

In the time course study of C2 cells (see Figure 3.05), KP372-1 at 400 nM initially down-regulated phosphorylation of mTORC1 substrates S6RP and 4E-BP1, and then gradually down-regulated phosphorylation of Akt and eIF4E. Finally, no protein was available from the KP372-1-treated cells. Besides, Annexin V/PI staining showed that 400 nM KP372-1 induced late apoptosis in most C2 cells after 24 hours of incubation (Figure 3.07A and 3.07B). Taken together, the experiments indicate that the phenomena of decreased phosphorylation and loss of protein may be correlated with apoptosis and/or the drug action of KP372-1. In addition to Akt, KP372-1 has been reported to target PDK1 and Flt3, a RTK (Zeng *et al.* 2006). Thus it could be inferred that the profound inhibition by KP372-1 of Akt and eIF4E phosphorylation in the present study is mediated by these two additional targets. However, whilst PDK1 inhibition may account for the reduced Akt phosphorylation, it is more difficult to explain the observed decrease in eIF4E phosphorylation. First, accumulating evidence has shown that Erk and p38 mitogen-activated protein kinase (p38MAPK)/Mnk-mediated phosphorylation of eIF4E on Ser209 is critical for tumourigenicity but not essential for normal cell development. Down-regulation of Mnk-mediated eIF4E phosphorylation can be achieved through simultaneous inhibition of both Erk and p38MAPK (Ueda *et al.* 2004; Bianchini *et al.* 2008). In the present study, the combination of Rapamycin and pan-class I PI3K, including Wortmannin and ZSTK474 (see Figure 3.12 and 3.13), shows that full inhibition of 4E-BP1 phosphorylation fails to down-regulate eIF4E activity in all cell lines, in agreement with previous findings that both mTORC1 and MAPK pathways concurrently regulate eIF4E activity (Raught and Gingras 1999). Second, in a study of human throid cancer cells *in vitro*, KP372-1 produced no inhibitory effect on MAPK phosphorylation (Mandal *et al.* 2005). Although

KP372-1 simultaneously inhibits Akt, PDK1 and Flt3 in AML cells *in vitro*, no inhibition of ERK and PDK1 phosphorylation has been observed, suggesting that KP372-1 does not abrogate Flt3-mediated class I PI3K and Erk MAPK pathways but can down-regulate the Flt3-mediated Jak/STAT pathway (Zeng *et al.* 2006). Moreover, Flt3 is commonly expressed on precursor cells of myeloid and lymphoid lineages in bone marrow and less commonly on the hematopoietic system-associated organs, including liver, spleen, thymus and placenta. Expression of mutant Flt3 has been observed in seveal cancer types, all of which are confined to hematologic malignancies including B-cell or T-cell acute lymphoblastic leukemia and B-cell acute myeloid leukemia, myelodysplasia, chronic myeloid leukemia and chronic lymphocyctic leukemia (Meshinchi and Appelbaum 2009). Jurakt T and C2 cells, derived from human acute myeloblastic leukemia and canine mastocytoma respectively, have been reported as having low or lost expression of Flt3 (Lyman and Jacobsen 1998; Inomata *et al.* 2006). Canine REM134, J3T and SB cells, derived from canine mammary gland tumour, glioma, and hemangiosarcoma respectively, are considered to be unlikely to express Flt3, based on the cell origins of the three tumours derived from epithelial/mesenchymal, neural, and endothelial cells, respectively, instead of bone marrow cells (Moulton 1990). Only 3132 cells derived from B cell lymphoma is required for further investigation (Drexler 1996). Collectively, Western Blot analysis has shown that Erk signaling is active in all canine cell lines in the present study. KP372-1 has been reported to have no effects on inhibition of Erk and MAPK phosphorylation. Therefore, it is unlikely that KP372-1 inhibits Erk and p38MAPK/Mnk-mediated eIF4E phosphorylation (Zeng *et al.* 2006).

It has been documented that cells undergoing apoptosis, at the molecular level, are characterized by exposure of phosphatidylserine (PS) on the cell surface, mitochondrial changes and the release of cytochrome c from the inner membrane of mitochondria to cytosol, activation of caspase signaling cascades that result in DNA fragmentation, degradation of nuclear material and cytoskeletal proteins (Elmore 2007). During apoptosis, cells display morphological changes, including blebbing of

cytoplasmic membrane, chromatin condensation, nuclear degradation, disintegration of the cells into apoptotic bodies (Elmore 2007; Krysko *et al.* 2008). In a host body, apoptotic bodies are normally engulfed by phagocytes (Krysko *et al.* 2008). When cells are grown in medium in the absence of phagocytes, apoptotic cells proceed to secondary necrosis that are characterized by lack of cell membrane integrity and changes in cell membrane permeability, thereby allowing PI to intercalate with fragmented DNA and leakage of proteins in the cell medium (Kelly *et al.* 2003; Krysko *et al.* 2008). In the current study, the most possible explanation for the occurrence of protein loss in the KP372-1 treated C2 cell group may be attributed to the leakage of proteins from cytosol to cell medium during secondary necrosis, based on the observation of the majority (73-88%) of C2 cells being positive for both PI and Annexin V staining (Figure 3.05, 3.07A and 3.07B). The secondary necrosis-related leakage of proteins may also provide an explanation for the decreased levels of all the proteins, including Akt, S6RP, 4E-BP1 and eIF4E, in C2 cells. An alternative explanation for the down-regulation of these four phosphorylated proteins by KP372-1 may be attributed to the cleavage (or degradation) of Akt1 and Raf1 kinases by caspases. Over the past decade, a wide array of proteins has been identified as caspase substrates. Caspases can promote apoptosis through cleavage of their protein substrates (Earnshaw *et al.* 1999). An *in vitro* study showed that caspases inhibited class I PI3K/Akt and Raf/Erk pathways through cleavage of Raf-1 and Akt1. In the same study, Ras was identified as an additional target of caspases and cleavage of Ras by caspases shut down Ras activity (Widmann *et al.* 1998).

Taken together, the most likely scenario for the drug action of KP372-1 on C2 cells is that KP372-1 inhibits the levels of phosphorylated S6RP, 4E-BP1, and Akt. Meanwhile, KP372-1 promotes apoptosis, followed by activates caspase signaling cascades, cleaves Akt-1 and Raf-1 proteins in C2 cells, inhibits both class I PI3K/Akt and Raf/Erk signaling cascades, and thereby decreased phosphorylation levels of Akt and eIF4E. With apoptosis proceeding to secondary necrosis, there are changes in the permeability of C2 cell membrane, which results in leakage of proteins to cell medium and allowing propidium iodide to enter cells and intercalate with fragmented DNA.

Like C2 cells, KP372-1 decreased levels of phosphorylated Akt, S6RP, 4E-BP1 and eIF4E in REM134 and J3T cells. Besides, REM134 cells, as an adherent cell line, were observed to float in the medium following high dose of KP372-1 treatment for 5 hours and subsequently far less amount of protein was available as compared with the control. By contrast, the phenomena observed in REM cells did not happen to J3T cells (Figure 3.03C). It was observed that 92% of REM134 cells, 65% of J3T cells, and 31% of C2 cells were apoptotic after 5 hour incubation with KP372-1 (Figure 3.08). A majority of adherent cells, including REM134, J3T and SB cells, detached from the monolayer to float in the medium following 24 hour incubation with KP372-1. Taken together, it is speculated that the scenario of the drug action of KP372-1 on C2 cells may occur in REM134 and J3T cells as well.

3.4.3.3 KP372-1 induces apoptosis through inhibition of PDK1/Akt, independent of Flt3 inhibition

Previous studies of AML cell lines / xenografts / patients showed that KP372-1 inhibited mutant Flt3-mediated over-expression of PIM (stands for proviral integration sites associated with murine leukemia virus–induced lymphomagenesis) kinase through Janus kinase (Jak)/ signal transducers and activators of transcription (Stat) signaling (Zeng *et al.* 2006). PIM kinase functions to promote cell survival and growth through PIM substrates - some of these substrates, such as Bad, 4E-BP1 and p70S6K, are also regulated by Akt (Fox *et al.* 2003; Yan *et al.* 2003; Aho *et al.* 2004; Chen *et al.* 2005; Nawijn *et al.* 2011). KP372-1 is reported to have little impact on normal Flt3-mediated PIM activity. Moreover, the remarkable effect of KP372-1 on apoptosis induction has "not" been shown to correlate with normal or mutant Flt3 expression (Zeng *et al.* 2006). This suggests that induction of apoptosis by KP372-1 occurs predominantly through inhibition of PDK1/Akt-mediated pro-survival and anti-apoptotic activities. In agreement with previous findings (Zeng *et al.* 2006), it seems that there is no correlation between sensitivity to apoptosis and level of Flt3 expression among all the cell lines tested in the current study.

3.4.3.4 Comparison of the effects of KP372-1 on Akt/mTORC1 inhibition in previous studies with those in the current study

In the present study, KP372-1 down-regulates Akt/mTORC1 signaling in C2 cells after either short exposure to drug concentrations of ≥ IC-100 or prolonged exposure to lower doses. However, previous reports showed that lower doses (such as IC-50) of KP372-1 could suppress Akt/mTOR signaling in tumour cells cultured under different conditions. For example, in serum-free medium, short exposure (4-6 hours) to IC-50 concentrations of KP372-1 significantly decreased phosphorylation of Akt (Ser473) and S6RP in cells derived from human thyoid cancer and glioblastoma (Mandal *et al.* 2005; Koul *et al.* 2006). In a study of HNSCC, phosphorylation of Akt (Ser473) and S6RP was down-regulated in cells concomitantly incubated with IC-50 concentrations of KP372-1 and EGF for 30 mins, as compared with cells incubated with EGF only (Mandal *et al.* 2006). In the study of AML by Zeng *et al* (2006), the concentrations of IC-100 and lower than IC-100 of KP372-1 were utilized to treat cells, which were culture in serum-containing medium, for 8 to 16 hours. Notably, chronic exposure (16 hours) to IC-100 concentration of KP372-1 abrogated phosphorylation of Akt (Thr308 and Ser473) but had no inhibitory effect on Erk signaling in AML cells. Moreover, it was observed that phosphorylation of Akt on Ser473 was more sensitive to KP372-1 than Thr308, in that lower doses of KP372-1 could inhibit phosphorylation on Ser473 but not on Thr308 (Zeng *et al.* 2006). Taken together, an explanation for the requirement for longer drug exposure and higher concentration to inhibit Akt/mTORC1 signaling in the cells tested in the current study could possibly be due to cells cultured in serum-containing medium, which allows serum that may contain unidentified growth factors to constantly activate class I PI3K pathway in these cells.

It has been shown that levels of phosphorylated Akt (Ser473) were decreased to varying degrees in KP372-1-treated human HNSCC, thyroid and AML cells (Mandal *et al.* 2005; Mandal *et al.* 2006; Zeng *et al.* 2006). In contrast to previous findings, KP372-1 did not abrogate phosphorylation of Akt on Ser473 in human Jurkat T, canine 3132 and SB cells. This disparity in the blockade of Akt phosphorylation could be a

consequence of different drug treatment protocols. For example, HNSCC and thyroid cancer cells were treated with this inhibitor under serum-free condition whereas AML cells which were grown in serum-containing medium had been exposed to KP372-1 for 16 hours before harvested for Western blotting analysis (Mandal *et al.* 2005; Mandal *et al.* 2006; Zeng *et al.* 2006). In the current study, since Akt phosphorylation in C2 cells was abrogated after chronic exposure to KP372-1, it is speculated that Jurkat T, 3132 and SB cells might follow the same track as C2 cells. Moreover, the failure of KP372-1 to inhibit Akt phosphorylation in these cell lines suggests that PDK1 may not be the first priority target of KP373-1. However, to elucidate this issue whether PDK1 activity or Akt phosphorylation can be inhibited in Jurkat T, 3132 and SB cells, prolonged exposure to KP372-1, along with Western blotting analysis of phosphorylation of Akt on both Thr308 and Thr473, may provide a better understanding of the drug mechanism.

The exact mechanism by which KP372-1 inhibits Akt kinase activity remains unresolved. Other Akt inhibitors have been developed, such as AKTide-2T (which competes with substrate binding in the kinase (catalytic) domain), TCN/API-2 (which interacts with PH domain), and Akti-1,2 (an allosteric, non-competitive inhibitor which alters the overall shape of Akt kinase) (Cheng *et al.* 2005). So far, a competitive ATP inhibitor which specifically targets Akt has not been yet developed because Akt shares similar three dimensional structure of ATP-binding pocket with PKA and PKC (Lindsley *et al.* 2007).

3.4.3.5 Pharmacokinetic properties of KP372-1

KP372-1 is a recently-synthesized inhibitor and, at the time of writing, only data from *in vitro* experiments have been published. An *in vitro* study demonstrated dose-dependent induction of apoptosis for AML cells by KP372-1, whilst normal hematopoietic progenitors were unaffected/spared. The same study shows lesser inhibitory effects on clonogenic growth of normal bone marrow cells, as compared with that of AML cells (Zeng *et al.* 2006). In line with this, much less cytotoxic effects of

KP372-1 on normal human astrocytes and fibroblasts than those on glioblastoma cell lines has been reported in another study of human glioblastomas (Koul *et al*. 2006).

3.4.4 Rapamycin

3.4.4.1 Rapamycin triggers eIF4E survival pathways in certain cell lines

In this study, all canine cell lines exhibited relative resistance to Rapamycin, as indicated by less than 50% inhibition of viability at the dose (~600 nM) recommended for clinical treatment (Kwitkowski *et al.* 2010). Further, Rapamycin at the doses lower than 10 μM showed only cytostatic effects on cell viability in these canine cells whereas remarkable reduction in cell viability was observed at doses of 10-40 μM, in agreement with previous findings that micromolar ranges of CCI-779 or Rapamycin could achieve full inhibition of cell proliferation (Shor *et al.* 2008). Accumulating evidence suggests that Rapamycin at lower doses (nanomolar range) requires initial interaction with the cytoplasmic receptor FKBP12, which in turn allows Rapamycin to bind mTORC1, leading to inhibition of mTORC1 pathway but also generation of drug resistance issue (Shor *et al.* 2008; Chen *et al.* 2010). At least three mechanisms have been reported as giving rise to Rapamycin resistance, and all of them are linked to mTORC1 inhibition. The first is through inhibition of mTORC1/p70S6K, which in turn releases the feedback loop of p70S6K/IRS-1/PI3K/Ras and stimulates Ras/Erk MAPK and PI3K/Akt pathways (Harrington *et al.* 2004; Wang *et al.* 2007; Carracedo *et al.* 2008). For the second, inhibition of mTORC1 stimulates expression of insulin growth factor (IGF-1) and IRS-2, followed by activation of IGF-1/IGF-1 RTK/IRS-2/PI3K with consequent stimulation of the class I PI3K/Akt pathway (Tamburini *et al.* 2008). The third route is through mTORC1 inhibition, which triggers the c-SRC/RTK pathway and causes activation of Ras/Erk MAPK pathway (Chaturvedi *et al.* 2009). By contrast, Rapamycin at higher doses (micromolar range) directly binds mTOR, which in turn inhibits mTORC2, mTORC1 and global translation process, leading to dramatic decline in cell viability (Shor *et al.* 2008). A more recent study confirmed that high concentrations of

Rapamycin inhibited both mTORC1 and mTORC2 and had better efficacy in eradiaction of tumour cells than low concentrations of Rapamycin. Rapamycin (at high concentrations)-mediated mTORC2 inhibition causes a reduction in phosphorylation levels of Akt and Erk (Chen *et al.* 2010).

In agreement with previous findings (Shor *et al.* 2008), the current Western blot results show that a low dose (100 nM) of Rapamycin inhibits mTORC1 signaling in all lines, as evidenced by decreased phosphorylation of either S6RP or 4E-BP1. However, it also stimulates phosphorylation of eIF4E in Jurkat T cells. As eIF4E phosphorylation is under the control of Erk and/or p38 MAPK pathways following mTORC1-mediated dissociation from 4E-BP1, these results suggest that Rapamycin at the low dose stimulates the Erk or p38MAPK/Mnk/eIF4E pathway in Jurkat T cells, possibly through one of the three Rapamycin-resistance mechanisms described above (Waskiewicz *et al.* 1997; Raught and Gingras 1999; Sun *et al.* 2005; Bianchini *et al.* 2008). Indeed, a previous study of PIM inhibitor demonstrated that inhibition of p70S6K activity in Jurkat T cells triggered p70S6K/IRS-1 feedback loop and activated Ras/Erk signaling (Lin *et al.* 2010). In this study, not only Rapamycin but also KP372-1 significantly increased phosphorylation of eIF4E in the tested cell lines. Interestingly, the Rapamycin-induced phosphorylation of eIF4E in Jurkat T cells was suppressed when cells were treated with combinations of Rapamycin with either ZSTK474 or Wortmannin. In fact, it was observed that Rapamycin up-regulated eIF4E phosphorylation in 3132 and SB cells and the effects lasted for 48-72 hours. eIF4E phosphorylation in SB cells was up-regulated by Rapamycin alone, Wortmannin alone and the combination of both inhibitors. As previous study reported that Rapamycin-induced eIF4E phosphorylation could be reversed by the combination of Rapamycin and PI3K inhibitor but, sometimes, PI3K inhibitor alone still increased eIF4E phosphorylation. Therefore, it is suggested that tumour cells may escape the drug treatment through additional mechanisms other than p70S6K/IRS-1/PI3K/Ras feedback loop (Wang *et al.* 2007). Consistent with previous findings, the data suggest that the resistance of Jurkat T, SB and 3132 cells to these PI3K pathway inhibitors is through more than one mechanism.

3.4.4.2 The possible mechanism for high doses of Rapamycin to induce apoptosis

Rapamycin at higher doses (micromolar range) was demonstrated to directly binds to mTOR, which in turn inactivated mTORC2 activity and inhibited mTORC1-independent protein synthesis mechanism, leading to a dramatic decline in cell viability (Shor *et al.* 2008). Besides, inhibition of mTORC2 by a high dose (1 μM) of Rapamycin was shown to increase apoptotic cell numbers and down-regulate Akt and Erk phosphorylation, as compared with the controls (Chen *et al.* 2010). In this study, Rapamycin at 20 μM significantly increased apoptosis for most of the tested cell lines, confirming that reduction of cell viability was in part through apoptosis.

To date, mTOR kinase is the only target of Rapamycin (Huang *et al.* 2003). It is speculated that Rapamycin-mediated apoptosis and inhibition of mTORC1-independent protein synthesis may be attributed to Rapamycin-mediated mTORC2 inhibition. mTORC2 has recently been identified as an activator of not only Akt survival kinase but also SGK, a pro-survival factor, and PKC which is involved in a variety of cellular physiological functions and tumourigenesis (Sarbassov *et al.* 2005; Garcia-Martinez and Alessi 2008; Ikenoue *et al.* 2008; Gonelli *et al.* 2009). More recently, mTORC2 has been found to play a role in regulation of Erk signalling through Rictor, a component of mTORC2. Previous studies showed that the role of Rictor in regulation of Erk pathway is cell-specific. In human MCF-7 (breast cancer) and DU145 (prostate cancer) cell lines, high concentrations of Rapamycin or silencing of Rictor mRNA inhibited Erk phosphorylation (Chen *et al.* 2010). By contrast, in glioma cell lines and glioma primary cells, silencing of Rictor mRNA induced Erk phosphorylation and showed no effect on inhibition of cell viability although Akt activity was impaired (Das *et al.* 2011). Taken together, it appears that the role of Rictor in activation or inhibition of Erk is cell line-dependent. Rictor regulates not only Erk but also Akt and some PKCs (Ikenoue *et al.* 2008; Chen *et al.* 2010; Das *et al.* 2011). Protor-1 regulates SGK (Pearce *et al.* 2011). The mechanism of high doses of Rapamycin-mediated apoptosis may rely on inhibition of mTORC2-dependent Akt, all conventional PKCs, novel PKCε and SGK survival

pathways. After mTORC2 inhibiton, Erk survival pathway may be inactivated and drive cells apoptosis, or may be stimulated and resistant to Rapamycin treatment.

3.4.5 Rapamycin combined with Wortmannin

In this study, the combination of Wortmannin and Rapmycin inhibited most cell lines in an additive or synergistic manner, except REM134. By utilizing Western blot analysis to further dissect the mechanism, it was observed that all cell lines presented similar Western blot profiles except the phosphorylation levels of Akt in the drug combination-treated cells (Figure 3.11). The combination of Wortmannin with Rapamycin significantly down-regulated Akt phosphorylation in most cell lines, including Jurkat T, 3132, SB and C2 cells, whereas this combination had no effects on REM134 and J3T cells (Figure 3.11). Therefore, it is suggested that the failure of the drug combination to down-regulate Akt phosphorylation in REM134 cells, leading to constitutive activation of Akt survival signaling, may provide an explanation for the antagonostic effects on this cell line. In line with this explanation, in J3T cells, there was not much difference in viability inhibition between Rapamycin alone and the drug combination groups (see Figure 3.09), suggesting a correlation between the degree of decreased levels of Akt phosphorylation and inhibitory efficacy of Wortmannin combined with Rapamycin.

However, the Western blot results cannot distinquish the difference between additive and synergistic effects of the drug combination. The discrepancy may be explained by multiple targets of Wortmannin. As mentioned above, Wortmannin has 20-50 fold selective inhibition of p110α catalytic subunit over PI4K and other PI3K-related kinases (Stein 2001). It is suggested that the synergistic effects may be the result of the effects on other targets of Wortmannin in addition to class I PI3K.

Although the drug combination profoundly inhibited 4E-BP1 phosphorylation, as compared with single drug-treated cells, this did not contribute to down-regulation of eIF4E activity across all cell lines (Figure 3.11). This is in agreement with previous

findings that eIF4E phosphorylation is concurrently regulated by mTORC1/4E-BP1 and Erk and p38MAPK/Mnk signaling pathways (Raught and Gingras 1999; Wang *et al.* 2007; Bianchini *et al.* 2008). Grzmil M *et al* (2011) reported that simultaneous inhibition of Mnk and mTOR kinases synergistically decreased growth of human glioblastoma cells *in vitro* (Grzmil *et al.* 2011). The same group described unpublished observations whereby concomitant inhibition of Mnk and mTOR kinases promoted the association of 4E-BP1 with eIF4E, suggesting that the Erk and p38 MAPK/Mnk and mTOR pathways co-regulated eIF4E phosphorylation and the cap-dependent translation. Since all the tested canine lines demonstrate active Erk signaling, it seems likely that constant eIF4E phosphorylation is sustained by active Erk. Although active Erk is not expressed in Jurkat T cells, it cannot be ruled out that Jurkat T cells might express active p38MAPK, which is another upstream activator of Mnk/eIF4E pathway (Ueda *et al.* 2004; Bianchini *et al.* 2008). To elucidate whether mTOR and Mnk concurrently regulate eIF4E activity in these cell lines, investigation of inhibitors specifically targeting the Erk and/or p38MAPK/Mnk pathway may give better understanding of eIF4E regulation.

3.4.6 Rapamycin combined with ZSTK474

It is suggested that the mechanism for the additive or synergistic effects of ZSTK474 and Rapamycin on cells occurs through simultaneous inhibition of Akt activity and mTORC1 activity. Like Wortmannin combined with Rapamycin, the combination of ZSTK474 and Rapamycin has no effects on eIF4E phosphorylation, consistent with previous reports that eIF4E phosphorylation was concurrently regulated by mTOR and Mnk pathways (Raught and Gingras 1999; Wang *et al.* 2007; Bianchini *et al.* 2008). Interestingly, it was observed that the combination of ZSTK474 and Rapamycin did not profoundly inhibit phosphorylation of S6RP in tested canine cell lines other than C2 cells. S6RP was reported to have three upstream activators - PDK1/p70S6K, mTORC1/p70S6K and Ras/Erk/RSK pathways. It is suggested that Ras/Erk/RSK is most likely to contribute to the maintenance of S6RP phosphorylation after blockade of both PI3K and mTORC1 signaling in these four canine cell lines

(Burnett *et al.* 1998; Pullen *et al.* 1998; Roux *et al.* 2007). As simultaneous inhibition of class I PI3K and mTOR by the drug combination can result in down-regulation of PDK1- and mTOR-mediated phosphorylation of PDK1, it is possible that the active Erk signaling which is detected in these canine cell lines supports S6RP activity. This would also explain the limited effects of Rapamycin on S6RP phosphorylation in some cell lines such as 3132.

Recently, simultaneous inhibition of class I PI3K/Akt and Raf/Mek/Erk signaling pathways has been proposed as a novel anti-cancer strategy, as mutant Ras may trigger both signaling pathways to maintain survival and proliferation of tumour cells. Moreover, cross-talk between class I PI3K and Mek pathways is the major resistance mechanism which attenuates efficacy of single agent targeting either PI3K or Mek signaling (Castellano and Downward 2011). Indeed, accumulating evidence from *in vitro* and *in vivo* studies has demonstrated that concomitant treatment with class I PI3K and Mek pathway inhibitors exerts additive effects on growth inhibition of tumour cells (Bianchini *et al.* 2008; Engelman *et al.* 2008). As the data presented here have revealed activation of both class I PI3K and Erk pathways in the tested canine cells, future strategies to effectively eliminate cell viability in canine tumours may utilize inhibitors co-targeting both pathways.

Although cells displayed variable responses to the combination of Wortmannin and Rapamycin, Wortmannin demonstrated synergism with Rapamycin in C2 and SB cells; by constrast, for J3T cells, ZSTK474 and Rapamycin acted synergistically. This suggests that the sensitivity to a given drug combination may vary from one cell line to another, presumably due to each cell line possessing its unique cellular context for survival and proliferation. Moreover, the results also implicate that each cancer patient may suit different combination of the PI3K pathway inhibitors to antagonize cancer progression.

In this study, it is observed that chronic exposure (18 hours) to Rapamycin down-regulates Akt phosphorylation in Jurkat T cells (Figure 3.12). The same effect of

Rapamycin on reducing Akt phosphorylation in Jurkat T cells and certain cell lines after exposure for 24 hours has been described in previous studies (Sarbassov *et al.* 2006; Dal Col *et al.* 2008). The present study shows that chronic exposure to Rapamycin down-regulates both mTORC1 signaling and Akt phosphorylation in Jurkat T cells, which may underlie the high sensitivity of Jurkat T cells to Rapamycin (Figure 3.02D).

3.4.7 Drug combination strategy – class I PI3K pathway inhibitors and Doxorubicin

In the current study (see Figure 3.13), cells showed variable responses to the combination of class I PI3K pathway inhibitors and Doxorubicin, a cytotoxic agent that intercalates DNA at synthesis (S) phase, interfering with DNA synthesis, and which also directly inhibits topoisomerase II activity (Momparler *et al.* 1976; Burgess *et al.* 2008). Interestingly, SB cells were the least sensitive to KP372-1 treatment but the combination of KP372-1 with Doxorubicin acted synergistically on this cell line whereas for REM134 cells, which were highly sensitive to KP372-1, the drug combination resulted in antagonism (Figure 3.02C and 3.13). One possible explanation for such results could be an unknown mechanism at the level of DNA synthesis (S phase) may antagonize KP372-1-mediated apoptosis in SB cells.

In the three cell lines REM134, 3132 and C2, the combination of Doxorubicin with the class I PI3K pathway inhibitors was antagonistic. Although accumulating evidence supports that inhibition of PI3K signaling pathway (with resultant cell cycle arrest and apoptosis induction), may enhance effects of Doxorubicin and other chemotherapeutic drugs, a small number of studies have reported that cells respond poorly to this drug combination. This may occur because sequential exposure to the PI3K inhibitors prior to (earlier than) Doxorubicin, leading to more cells resting in G0-G1 phase, thereby preventing Doxorubicin from binding to proliferative cancer cells (Opel *et al.* 2008; McDonald *et al.* 2010; Bender *et al.* 2011). As the canine cells were treated with Doxorubicin and the PI3K pathway inhibitors simultaneously, it is possible that Wortmannin, ZSTK474 and Rapamycin lead to accumulation of cells in G0-G1

phase, abrogating the efficacy of Doxorubicin. Previous data demonstrated that the mechanism of ZSTK474 to down-regulate cell viability was predominantly through inhibition of cell growth (Yaguchi *et al.* 2006). In line with the findings by Yaguchi *et al* (2006), the current data showed that 20 μM ZSTK474 significantly inhibited viability in all cell lines with a reduction in viability ≥79%. However, this inhibitor at 20 μM produced apoptosis with an increase in apoptotic fraction by ≤ 35% in Jurkat T, SB and C2 cells, and even had almost no apoptotic effects on 3132, REM134 and J3T cells (Figure 3.02B, 3.07A and 3.07B). Wortmannin and Rapamycin have been well-established for their ability to arrest cell cycling (De Nadai *et al.* 1998; Dal Col *et al.* 2008). Therefore, previous data, along with the current findings, may provide an explanation for the antagonistic effects of Doxorubicin in combination with the three class I PI3K pathway inhibitors Wortmannin, ZSTK474 and Rapamycin. To improve the efficacy of Doxorubicin combined with PI3K pathway inhibitors, cells could be pretreated with Doxorubicin for 24 hours, and then concomitantly treated with Doxorubicin and the PI3K pathway inhibitors for a further ≥ 48 hours. This approach might prevent cells from accumulating at G0/G1 phase and allow Doxorubicin to interact with more cells at S phase (McDonald *et al.* 2010).

In contrast to Wortmannin, ZSTK474 and Rapamycin, the mechanism for KP372-1 to reduce cell viability is preferentially through apoptosis induction (Zeng *et al.* 2006). This may explain the synergistic effects of the KP372-1 / Doxorubicin combination observed for SB cells. However, only data of the combination of KP372-1 and Doxorubicin are available from SB and REM134 cells. This is not enough to validate the theory that KP372-1 has less interference with Doxorubicin than the other three PI3K pathway inhibitors. To avoid the issue that class I PI3K pathway inhibitor-induced G0/G1 cell cycle arrest may conteract the efficacy of Doxorubicin, the approach of investigation into the effects of Doxorubicin in combination with these inhibitors on cancer cells should be performed by treating cells with Doxorubicin 24 hours prior to these inhibitors, instead of comcomitant treatment of two drugs for cells. Other factors that may influence the efficacy of the PI3K pathway inhibitors combined with Doxorubicin include genetic background and cellular context in a given cell line,

activation of the PI3K/Akt survival mechanism in response to Doxorubicin treatment, and other pharmacological properties such as potential interations between the lipid-soluble inhibitor and water-soluble Doxorubicin (Wang *et al.* 2009; Wallin *et al.* 2010). One solution to eliminate the interaction between lipid- and water-soluble drugs is to treat cells with both drugs which are water-soluble. For example, Temsirolimus is a water-soluble Rapamycin derivative (Kwitkowski *et al.* 2010).

Taken together, the true reasons that cause variable responses to the drug combination remain to be learned and one of the reasons could be cell-type-dependent. Nonetheless, few additive/synergistic effects of the drug combination cases still encourage the potential of integrating the PI3K pathway inhibitors into conventional chemotherapy protocol in canine oncology.

3.4.8 Autophagy

Under nutrient deprivation or stress condition, cells undergo either apoptosis or autophagy. Autophagy is a process that degrades proteins and organelles and then recycling macromolecules in order to maintain cell survival (Shintani and Klionsky 2004). The current data (see Figure 3.14B and 3.14C) show that prolonged exposure to Rapamycin for 48-72 hours, leads to increasd levels of LC3B-II, which is an indicator of autophagy induction, in canine SB, REM134 and J3T cell lines. This suggests that these drugs may potentiate the autophagy process to resist anti-cancer therapy. Recently, PP242, a novel inhibitor specifically targeting mTORC1 and mTORC2, has been reported to delay tumour onset and inhibit cancer cell growth more efficiently than Rapamycin. However, PP242 also showed greater potency to induce autophagy than Rapamycin (Janes *et al.* 2010). NVP-BEZ235, a newly developed inhibitor specifically targeting class I PI3K, mTORC1 and mTORC2 and currently enters clinical trials, is reported to induce autophagic resistance despite this inhibitor displaying promising activity against tumour *in vitro* (http://clinicaltrials.gov/ct2/search) (Baumann *et al.* 2009). One strategy currently under investigation is combined treatment with NVP-BEZ235 and the autophagic inhibitor Chloroquine; this approach is reported to enhance

the effect of NVP-BEZ235 on apoptotic induction (Chiarini *et al.* 2010; Fan *et al.* 2010; Xu *et al.* 2011). However, not all inhibitors targeting the class I PI3K/Akt/mTOR pathway can cooperate with autophagic inhibitors. For example, inhibition of autophagy process does not enhance the efficacy of Rapamycin towards glioma cells due to activation by Rapamcin of the Akt survival pathway (Fan *et al.* 2010).

One confounding issue in the current study is that induction of autophagy was observed in control cells as well as drug-treated cells. Vehicle-treated (control) J3T and REM134 cells expressed similar levels of LC3B-II to drug-treated groups after short exposure to ZSTK474 and/or Rapamycin (Figure 3.14A). Moreover, in the time course study over 72 hours, a higher level of LC3B-II expression was seen in vehicle-treated J3T cells as compared with Rapamycin-treated cells (Figure 3.14C). One possibility is that J3T and REM134 cells are more sensitive to stress stimuli in the environment, based on the consistent presence of LC3B-II expression in both lines after short-term or long-term cell culture. Standard tissue culture manipulations, including removal of cells from incubators when adding drugs, or scraping cells from plates, are likely to result in a degree of cellular stress. Another source of stress stimuli might come from fast proliferation rates of cells, which resulted in over-confluence of cells in culture plates and followed by nutrient deprevation and autophagy induction. This may provide an explanation for presence of autophagy induction in J3T cells following long hour cell culture. Thus, it appears vital that external stress stimuli should be minimized throughout the course of experiments investigating the autophagy process. There are some ways to reduce external stimuli to cells. The first way is to use a flask with 80-90% confluence of cells, instead of a flask with over-confluence of cells. Secondly, cells prefer to be cultured in the incubator at at 37 ^{o}C in a humidified atmosphere with 5% CO_2, rather than stay in the hood at room temperature. Therefore, to avoid leaving cells outside the incubator for a long period of time, the time for for adding drug(s) or vehicle control reagent, such as DMSO, to each experimental or control group should be shortened. Thirdly, because scraping cells from each flask cannot be completed in a short period of time, it is recommended to handle few samples (flasks) at the same time,

in order to avoid leaving cells on a lab bench too long causing cellular stress. The solutions provided here may reduce the possibility of autophagy induction in vehicle control, avoidthe misunderstanding of the drug effects on autophagic resistance, and assure the credibility of data interpretation. Besides, future work should include a positive control cell line such as human neuroblastoma SH-SY5Y cell line, when investigating the effect of Rapamycin on induction of autophagy (Pan *et al.* 2009).

With regard to 3132 cells, the data suggest that Rapamycin and ZSTK474 do not promote autophagy in 3132 cells (Figure 3.14D), as indicated by a lack of LC3B-II expression in drug-treated cells. Similar effects are also observed in Jurkat T cells, suggesting that this cell line does not undergo autophagic resistance to either Rapamycin/ZSTK474 alone or the combination of both drugs. This may provide an explanation for high sensitivity of Jurkat T cells to ZSTK474 and Rapamycin. Taken together, the current study suggests that Rapamycin- and ZSTK474-induced autophagy is cell type-specific.

An alternative explanation for increased LC3B-II expression, an indicator of autophagy induction, in vehicle control cells would be the quality of the antibody used for Western blot analysis. Thus it will be important to validate the Western blot analysis of LC3B-II expression in canine cell lines using other approaches. To further investigate the role of autophagy, transmission electron microscopy analysis for presence of autophagic vacuoles could be performed (Chiarini *et al.* 2010). Other techniques could include acridine orange staining or autofluorescent monodansylcadaverine staining (to detect autophagosomes or autophagic vacuoles), and analysis of expression of the autophagy marker Beclin using Western blot (Takeuchi *et al.* 2005; Chiarini *et al.* 2010). However, as genetic sequence of canine Beclin has not been published yet, to check sequence homology of Beclin gene between human and canine species is necessary before performing western blotting analysis of Beclin expression in canine cells.

It appears that the canine cancer cell lines, with the exception of 3132 cells, may develop autophagic resistance in response to Rapamycin or ZSTK474. Although

previous studies have offered a solution to overcome autophagic resistance to some novel class I PI3K pathway inhibitors by concomitant treatment with the autophagy inhibitor Chloroquine, this may not prove effective with Rapamycin, due to Rapamycin-mediated up-regulation of Akt survival pathway in certain cell lines (Fan *et al.* 2010). Conversely, Chloroquine may enhance the efficacy of ZSTK474 which promotes down-regulation of Akt activity. However, this theory should be validated by the data obtained from pratical work.

Chapter 4: Expression profiling of class I PI3K signaling network of cancer stem cells and parental cells from canine glioma

4.1 Abstract

Growing evidence has demonstrated the existence of cancer stem cells (CSCs) within a variety of primary tumours and tumour cell lines and the critical role of CSCs in recapitulating a new tumour similar to its parent tumour. Moreover, many studies have shown the correlation of class I PI3K/Akt/mTOR signaling with the viability and maintenance of CSCs. In this study, we have successfully isolated putative CSCs from canine glioma J3T cells by neurosphere formation assay and identified several highly up-regulated genes, particularly IGFBP2 (27-fold), FYN (9.3-fold), and DDIT4 (8.5-fold), in the CSCs by cDNA microarray analysis. In the J3T CSCs, the genes encoding components of class I PI3K/Akt/mTOR axis pathway either remain unchanged or down-regulated. The majority of the genes encoding components of mTORC1/eIF4E pathway-modulated translation-initiation machinery are down-regulated. Ingenuity Pathways Analysis of the entire differentially expressed genes between the J3T CSCs and their parental cells indicate that the cell cycle, genetic disorder and protein synthesis vary the greatest between the two cell groups. In conclusion, the cDNA microarray data suggest that the stem cell biology of J3T CSCs are independent of class I PI3K/Akt/mTOR axis pathway.

4.2 Introduction

Gliomas are the most frequent primary brain tumours in human adults as well as certain dog breeds including Boxers and Boston Terriers (Stoica *et al.* 2011). High-grade gliomas, especially the most malignant GBM, display highly aggressive behavior and locally infiltrative nature, which often leads to incomplete surgical excision and tumour relapse. Current therapeutic regime by using the combination of surgery/radiotherapy/chemotherapy prolongs medium survival time of 12-15 months in human patients with glioblastoma multiforme (GBM) (Stupp *et al.* 2005; Wen and Kesari 2008). Dogs with astrocytic gliomas were reported to have maximal survival time of ~8 months after receiving chemotherapy alone or combined with radiotherapy and

surgery (Stoica *et al.* 2011). Therefore, it is suggested that poor prognosis continue to exist in the majority of human and dogs with high-grade malignant gliomas, which highlights the urgent need to develop new strategies for effectively controlling this disease.

There is growing evidence to support CSC theory based on the fact of tumour heterogeneity, a single tumour containing multiple tumour subpopulations that display diverse biological properties (Heppner 1984). The CSC theory states that a bulk of tumour mass is derived from few tumour cells that possess Stem Cell (SC) properties, including self-renewal, pluripotent/multipotent differentiation potential, unlimited replication and resistance to radiotherapy and chemotherapy. Like normal SCs that can build a whole organized organ/tissue, CSCs which are considered as transformed SCs are capable of self-renewing, differentiating and replicating in an uncontrolled manner, thus ultimately constructing an aberrant organ/tissue. As SCs express ABC transporters to exclude chemical compounds and are frequently at quiescent state, which contributes to SCs being resistance to chemotherapeutic agents and radiation, it is hypothesized that SCs may confer these resistant mechanisms to CSCs, which may account for the unresponsiveness of some tumours to chemotherapy/radiotherapy or relapse of tumours after chemotherapy/radiotherapy (Reya *et al.* 2001; Sausville and Longo 2001; Bunting 2002; Diehn *et al.* 2009).

To date, increasing evidence shows that class I PI3K/Akt/mTOR signaling pathway is associated with CSC biology and tumourigenesis. Studies showed that insertion of a myristolayted (constitutively active) Akt1 or inactivation of PTEN in hematopoietic stem cells (HSCs), which resulted in HSCs with hyperactive class I PI3K/Akt/mTOR signaling, induced transient cycling and proliferation of HSCs, but subsequently decreased HSC pool. Besides, hyperactivation of this pathway induced transformation of HSCs and initiated leukemia and other hematological malignancies (Zhang *et al.* 2006; Kharas *et al.* 2010). Reports of CSCs derived from human prostate, brain and breast cancers and preneoplastic mouse liver demonstrated that active class I PI3K/Akt/mTOR signaling contributed to survival and proliferation of CSCs and up-

regualtion of the activity of ABCG2, a multi-drug resistant ABC transporter (Zhou *et al.* 2007; Bleau *et al.* 2009; Dubrovska *et al.* 2009; Rountree *et al.* 2009).

In this study, the CSCs derived from canine glioma J3T cells were isolated and capable of proliferation in sphere formation assay. Subsequently, cDNA microarray analysis of J3T spheres versus their parental cells revealed differential expression of genes encoding several signaling pathways such as class I PI3K and Raf. Notably, several transcripts encoding components of class I PI3K/Akt/mTOR core signaling were down-regulated.

4.3 Results

4.3.1 Canine J3T cells form neurosphere colonies

Serum-free N2 medium supplemented with growth factors (EGF and bFGF), which favors the expansion of stem cell colony, has been established for the identification of neurospheres from mouse brain tissue and human brain tumours (Reynolds and Weiss 1992; Singh *et al.* 2003). In this study, J3T cells derived from canine glioma were observed to proliferate and form floated neurosphere-like clusters in a clonogenic manner. The materials and methods to grow J3T spheres were described in Chapter 2, Section 2.2. Like neurospheres derived from mouse brain tissue, the spheres from J3T cells had a spheroid appearance (Figure 4.01) (Reynolds and Weiss 1992) .

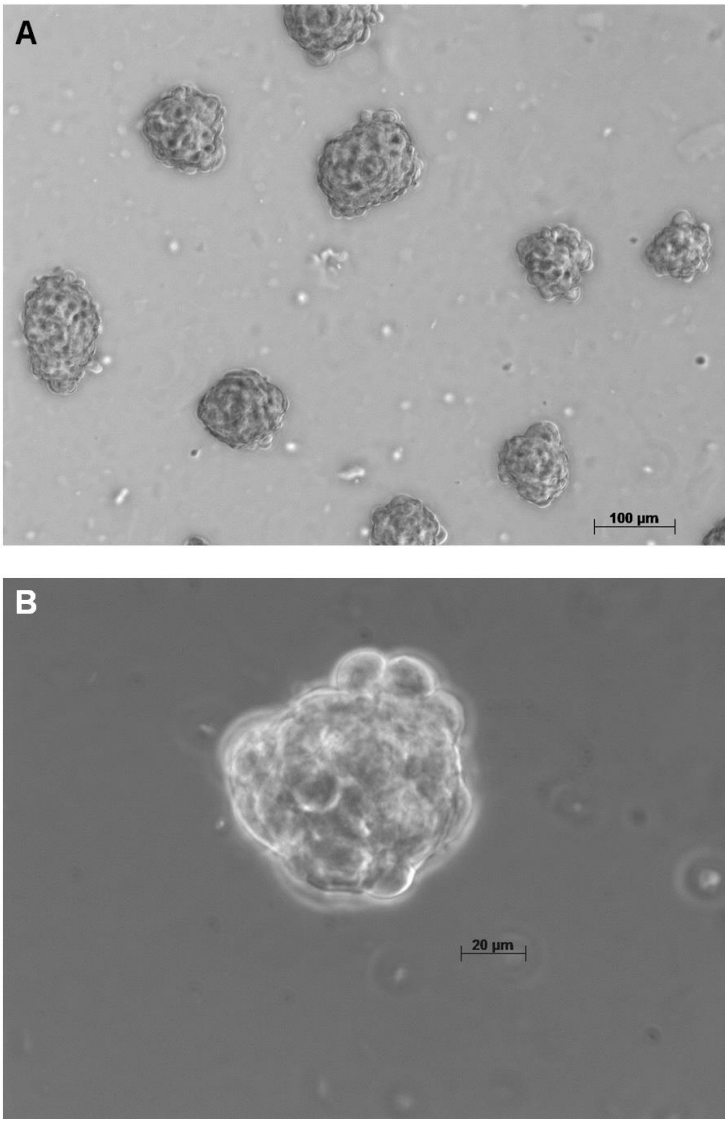


Figure 4.01. J3T cells formed nonadherent spheres under neural SC culture condition. Photomicrographs of cultured J3T cells were taken on Day 5 after plating in N2 medium with EGF and bFGF (the materials and methods described in Chapter 2, Section 2.2). The magnification of photomicrographs A and B of neurosphere-like cells was 10X and 40X, respectively. The photos courtesy of Dr. Lisa Pang.

4.3.2 Expression profiles of class I PI3K signaling network of J3T sphere cells

To identify genes that are differentially expressed in cells grown in sphere formation culture system compared with their parental cells grown in adherent monolayer culture, microarray analysis of gene expression profile of the two cell types derived from J3T cells by using Genechip Canine 2.0 Genome Arrays was performed and compared (the materials and methods being decribed in Chapter 2, Section 2.9). The fold change (or ratio) of each gene was obtained by comparison of the average value of the gene from J3T spheres with that from J3T parental cells. Analysis of the expression profiles of the genes encoding components of class I PI3K/Akt/mTOR axis pathway, its downstream p70S6K pathway and its main upstream regulators that are IR and IGF-1 pathways identified 73 genes being up-regulated and 59 genes being down-regulated (Table 4.01 and Table 4.02). Among the 73 up-regulated genes, IGFBP2 was the most up-regulated gene with a fold change of 27-fold. Besides, there were the other 11 genes which up-regulated $\geq$ 3-fold, including FYN, DDIT4, PPP1R3C, NTRK2, FOS, PDGFRB, CBL, IGF2R, CTNNB1, PLD3 and NTRK3 (Table 4.01). With regard to the 59 down-regulated genes, YWHAG was the most down-regulated gene with a fold change of 3-fold (Table 4.02).

It was observed that the key components of class I PI3K/Akt/mTOR axis pathway, including PIK3CD (encoding p110δ catalytic subunit of class I PI3K), PIK3R5 (p101 regulatory subunit of class I PI3K), AKT1 and EIF4E transcripts, were down-regulated (Table 4.02). Twenty-two transcripts encoding proteins involved in regulation of translation initiation were down-regulated, which included several subunits or isoforms of eukaryotic translation initiation factor (eIF)-1 (eIF1), X chromosome-linked eIF1A, eIF2, eIF2A, eIF2B, eIF2C, eIF3, eIF4A, and the protein product of PABPC1 gene (Table 4.02). On the contrary, five transcripts which were associated with

translation initiation were found to be up-regulated, including EIF1, EIF2C1, EIF2C3, PAIP1 and PAIP2 (Table 4.01).

In order to investigate the relationship of these genes categorized into class I PI3K pathway and its associated network, Ingenuity Pathways Analysis (IPA) was used to generate graphics of class I PI3K signaling network with showing differentially up-regulated and down-regulated genes (the method described in Chpater 2, Section 2.9.3). As shown in Figure 4.02, an overview of graphics of IR, IGF-1, class I PI3K/Akt, PTEN, mTOR, and regulation of eIF4 and p70S6K signaling pathways suggested that both class I PI3K/Akt/mTOR core signaling and eIF4E-mediated cap-dependent translation were down-regulated in J3T spheres. Additional information obtained from these graphics suggested that IR and most of its adaptor proteins including Cbl, Gab1, IRS-1, Jak1/2, and Shc were up-regulated (Figure 4.02A). IGFBPs were up-regulated but IGF-1 receptors (IGF-1Rs) were down-regulated (Figure 4.02B). With regard to those IR and IGF-1Rs-regulated downstream pathways in addition to class I PI3K signaling, Raf1/Mek1/Erk and Jak2/signal transducers and activators of transcription 3 (Stat3) pathways were up-regulated (Figure 4.02 A and B).

To further investigate the differences between the J3T spheres and their parental cells, IPA was employed to identify which canonical pathways and biological functions are relevant to these differentially expressed genes. As shown in Figure 4.03, the most relevant canonical pathways containing differentially expressed genes between the J3T spheres and their parental cells were ranked by statistical significance. It could be clearly seen that the majority of the top 17 canonical pathways were involved in regulation of protein synthesis and cell cycle progression. The regulation of p70S6K and eIF4E pathway, IGF-1R and PTEN pathways were ranked at top 8, 13 and 17, respectively (Figure 4.03). The most relevant (top 10) biological functions and diseases enriched with these differentially expressed genes between the J3T spheres and the J3T parental cells were related to cell cycle, genetic disorder, protein synthesis, cell death, cancer, post-transcriptional modification, DNA synthesis and repair, and post-translational

modification (Figure 4.04). The majority of these functions and diseases are involved in the tumourigenesis and cancer development.

Table 4.01. Ranking of the up-regulated genes encoding member proteins of class I PI3K pathway in J3T spheres, as compared with J3T parental cells. ANOVA was performed to determine significant differences ($p < 0.005$). The table courtesy of Dr. Lisa Pang.

Symbol	Entrez Gene Name	p-value	Fold Change	Entrez Gene ID for Human
IGFBP2	Insulin-like growth factor-binding protein 2	3.41E-13	27.128	3485
FYN	FYN oncogene related to FGR, SRC, YES	1.48E-13	9.334	2534
DDIT4	DNA-damage-inducible transcript 4	2.99E-07	8.506	54541
PPP1R3C	Protein phosphatase 1, regulatory subunit 3C	2.82E-05	7.969	5507
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	6.71E-13	7.805	4915
FOS	G0/G1 switch regulatory protein 7	1.39E-09	7.450	2353
PDGFRB	Platelet-derived growth factor receptor, beta polypeptide	1.14E-07	7.251	5159
CBL	Cas-Br-M murine ecotropic retroviral transforming sequence (c-CBL)	2.08E-12	5.566	867
IGF2R	Insulin-like growth factor 2 receptor	3.62E-07	4.862	3482
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	2.72E-15	4.145	1499
PLD3	Phospholipase D family, member 3	3.89E-07	3.925	23646
NTRK3	Neurotrophic tyrosine kinase, receptor, type 3	5.79E-08	3.138	4916
IGFBP7	Insulin-like growth factor-binding protein 7	9.15E-14	2.991	3490
IGFBP4	Insulin-like growth factor-binding protein 4	3.11E-11	2.852	3487
GAB1	GRB2-associated binding protein 1	5.07E-05	2.444	2549
JAK2	Janus kinase 2	1.02E-05	2.405	3717
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	5.84E-04	2.371	5156
ACCN1	Amiloride-sensitive cation channel neuronal 1	3.83E-05	2.253	40
RHOQ	Ras homolog gene family, member Q	5.58E-08	2.132	23433
FOXO1	Forkhead box O1	9.61E-07	2.116	2308
SOCS5	Suppressor of cytokine signaling 5	1.71E-06	2.045	9655
CDKN1A	Cyclin-dependent kinase inhibitor 1A	7.86E-07	2.040	1026
STX4	Syntaxin 4	5.28E-09	1.974	6810
RASA1	RAS p21 protein activator 1	1.65E-08	1.921	5921

Table 4.01. Ranking of the up-regulated genes encoding member proteins of class I PI3K pathway in J3T spheres, as compared with J3T parental cells. ANOVA was performed to determine significant differences ($p < 0.005$). The table courtesy of Dr. Lisa Pang. ***Continued***

Symbol	Entrez Gene Name	p-value	Fold Change	Entrez Gene ID for Human
FGFR1	Fibroblast growth factor receptor 1	2.33E-05	1.920	2260
BCL2	B-cell CLL/lymphoma 2	1.60E-04	1.918	596
FNBP1	Formin binding protein 1	9.19E-06	1.874	23048
RND3	Rho family GTPase 3	3.92E-05	1.863	390
PRKCD	Protein kinase C, delta	3.24E-07	1.835	5580
IGFBP6	Insulin-like growth factor-binding protein 6	6.03E-06	1.807	3489
CCND1	Cyclin D1	3.92E-06	1.799	595
RRAS	Related RAS viral (r-ras) oncogene homolog	1.76E-07	1.770	6237
PAIP2	Poly(A) binding protein interacting protein 2	5.59E-06	1.764	51247
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	1.14E-05	1.764	3791
EIF2C1	Eukaryotic translation initiation factor 2C, 1	8.66E-07	1.763	26523
NEDD4	Neural precursor cell expressed, developmentally down-regulated 4	5.43E-06	1.750	4734
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	1.22E-08	1.749	5573
INSR	Insulin receptor	3.83E-04	1.747	3643
PDGFC	Platelet derived growth factor C	9.35E-06	1.743	56034
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	2.34E-07	1.734	4170
SOCS2	Soc-2 suppressor of clear homolog (C. elegans)	5.88E-04	1.705	8835
TRIP10	Thyroid hormone receptor interactor 10	8.66E-06	1.703	9322
DDR1	Discoidin domain receptor tyrosine kinase 1	1.16E-05	1.673	780
RAF1	v-Raf-1 murine leukemia viral oncogene homolog 1	6.66E-07	1.659	5894
PIK3C3	Phosphoinositide-3-kinase, class 3	4.93E-07	1.619	5289
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	6.43E-06	1.603	6774
ACLY	ATP citrate lyase	1.38E-06	1.592	47
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	5.75E-04	1.573	3678
CNKSR3	Connector enhancer of kinase suppressor of ras 3	3.06E-04	1.555	154043

Table 4.01. Ranking of the up-regulated genes encoding member proteins of class I PI3K pathway in J3T spheres, as compared with J3T parental cells. ANOVA was performed to determine significant differences ($p < 0.005$). The table courtesy of Dr. Lisa Pang. ***Continued***

Symbol	Entrez Gene Name	p-value	Fold Change	Entrez Gene ID for Human
RHOT1	Ras homolog gene family, member T1	3.60E-08	1.540	55288
BCL2L1	BCL2-like 1	1.10E-06	1.536	598
RHOC	Ras homolog gene family, member C	1.56E-05	1.477	389
INPPL1	Inositol polyphosphate phosphatase-like 1	8.18E-06	1.473	3636
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	7.03E-07	1.465	5879
MAPK1	Mitogen-activated protein kinase 1	7.06E-05	1.461	5594
EIF1	Eukaryotic translation initiation factor 1	6.27E-06	1.442	10209
EIF2C3	Eukaryotic translation initiation factor 2C, 3	4.30E-04	1.438	192669
GHR	Growth hormone receptor	2.29E-04	1.426	2690
JUN	Jun proto-oncogene	2.85E-04	1.395	3725
GSK3B	Glycogen synthase kinase 3 beta	8.44E-04	1.381	2932
CSNK2A2	Casein kinase 2, alpha prime polypeptide	5.52E-05	1.357	1459
PAIP1	Poly(A) binding protein interacting protein 1	2.77E-07	1.345	10605
ITGB1	Integrin, beta 1 ibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12	2.84E-05	1.334	3688
STK11	Serine/threonine kinase 11	9.84E-05	1.320	6794
SOCS6	Suppressor of cytokine signaling 6	3.61E-04	1.319	9306
MAP2K1	Mitogen-activated protein kinase kinase 1	8.29E-06	1.308	5604
RHOA	Ras homolog gene family, member A	6.05E-08	1.300	387
IRS1	Insulin receptor substrate 1	1.20E-03	1.292	3667
PRKD3	Protein kinase D3	3.67E-04	1.265	23683
PPP2CB	Protein phosphatase 2, catalytic subunit, beta isozyme	3.25E-06	1.235	5516
FKBP1A	FK506 binding protein 1A, 12kDa	4.43E-04	1.172	2280
CYR61	Cysteine-rich, angiogenic inducer, 61	1.07E-03	1.171	3491
SHC1	SHC (Src homology 2 domain containing) transforming protein 1	4.94E-04	1.098	6464

Table 4.02. Ranking of the down-regulated genes encoding member proteins of class I PI3K pathway in J3T spheres, as compared with J3T parental cells. ANOVA was performed to determine significant differences ($p < 0.005$). The table courtesy of Dr. Lisa Pang.

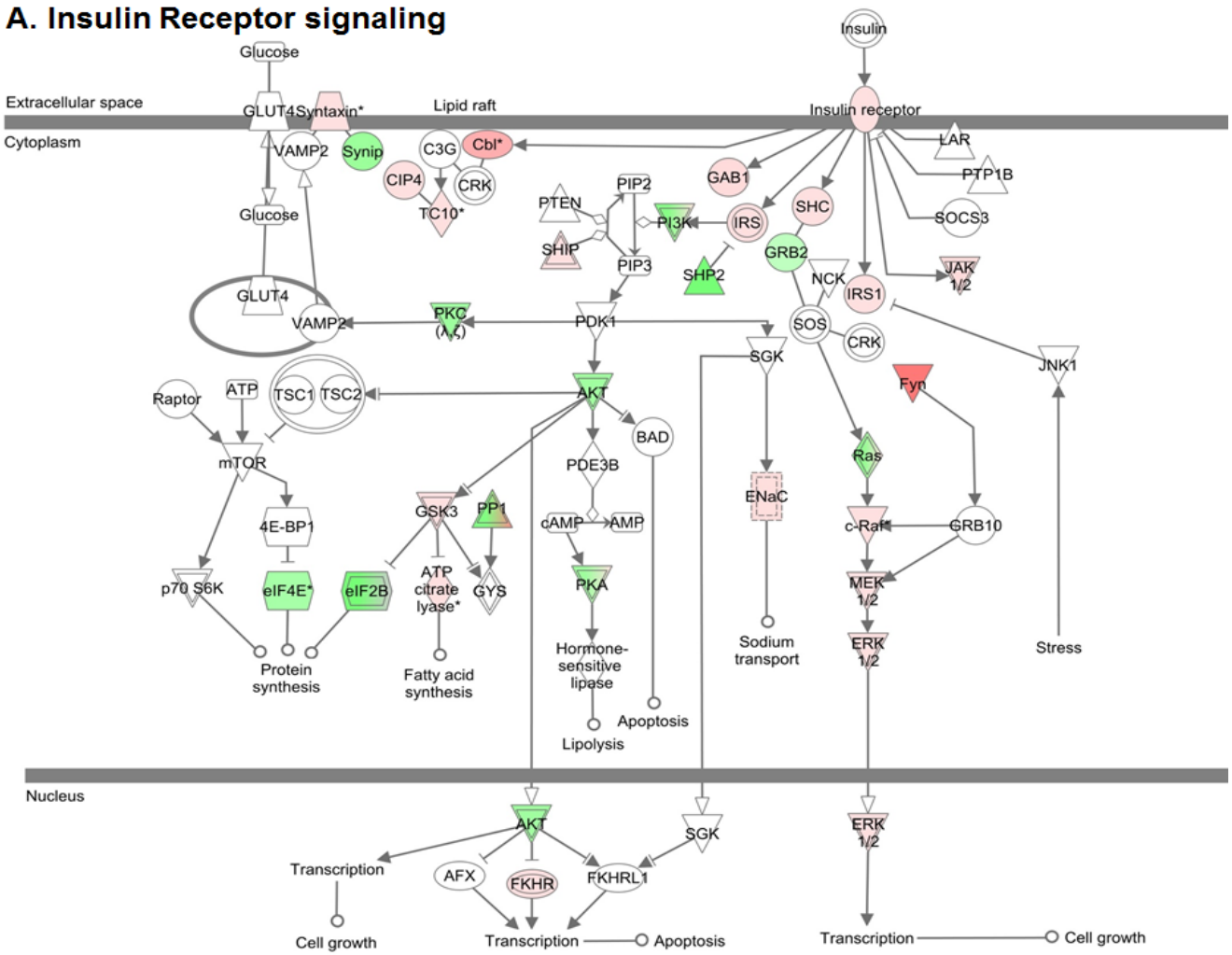
Symbol	Entrez Gene Name	p-value	Fold Change (-) indicates down-regulated	Entrez Gene ID for Human
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	2.63E-09	-3.020	7532
EIF2B2	Eukaryotic translation initiation factor 2B, subunit 2 beta, 39kDa	7.01E-07	-2.551	8892
PTPN11	Protein tyrosine phosphatase, non-receptor type 11	3.63E-08	-2.548	5781
PPP1R14B	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	1.48E-08	-2.333	26472
EIF2S1	Eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	4.15E-07	-2.288	1965
EIF3B	Eukaryotic translation initiation factor 3, subunit B	1.65E-11	-2.253	8662
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	7.52E-04	-2.243	5293
PTK2	PTK2 protein tyrosine kinase 2	1.78E-09	-2.200	5747
FGFR2	Fibroblast growth factor receptor 2	2.29E-09	-2.200	2263
EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa	3.05E-07	-2.190	1967
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	4.57E-06	-2.151	3845
THEM4	Thioesterase superfamily member 4	1.19E-07	-2.129	117145
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	1.81E-05	-2.106	2321
EIF3J	Eukaryotic translation initiation factor 3, subunit J	1.21E-12	-2.040	8669
EIF2B3	Eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	2.03E-08	-1.926	8891
EIF4A1	Eukaryotic translation initiation factor 4A1	6.48E-11	-1.871	1973
STXBP4	Syntaxin binding protein 4	3.11E-04	-1.855	252983
PRKCI	Protein kinase C, iota	7.03E-06	-1.822	5584
PRKAR2A	Protein kinase, cAMP-dependent, regulatory, type II, alpha	2.47E-07	-1.790	5576
AKT1	v-Akt murine thymoma viral oncogene homolog 1	1.39E-04	-1.760	207

Table 4.02. Ranking of the down-regulated genes encoding member proteins of class I PI3K pathway in J3T spheres, as compared with J3T parental cells. ANOVA was performed to determine significant differences ($p < 0.005$). The table courtesy of Dr. Lisa Pang. ***Continued***

Symbol	Entrez Gene Name	p-value	Fold Change (-) indicates down-regulated	Entrez Gene ID for Human
EIF1AX	Eukaryotic translation initiation factor 1A, X-linked	2.59E-10	-1.714	1964
EIF3D	Eukaryotic translation initiation factor 3, subunit D	9.24E-06	-1.696	8664
EIF3E	Eukaryotic translation initiation factor 3, subunit E	8.95E-06	-1.672	3646
EIF4E	Eukaryotic translation initiation factor 4E	1.86E-07	-1.654	1977
RPS6KA2	Ribosomal protein S6 kinase, 90kDa, polypeptide 2	4.19E-06	-1.589	6196
PPP2R3B	Protein phosphatase 2, regulatory subunit B'', beta	4.57E-05	-1.583	28227
PPP1R7	Protein phosphatase 1, regulatory (inhibitor) subunit 7	1.13E-05	-1.575	5510
EIF3A	Eukaryotic translation initiation factor 3, subunit A	1.54E-07	-1.572	8661
RHOJ	Ras homolog gene family, member J	3.75E-05	-1.559	57381
IGF1R	Insulin-like growth factor 1 receptor	9.62E-08	-1.554	3480
EIF3I	Eukaryotic translation initiation factor 3, subunit I	1.95E-05	-1.515	8668
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	2.45E-05	-1.495	7048
EIF2B4	Eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa	5.39E-09	-1.459	8890
MRAS	Muscle RAS oncogene homolog	1.14E-04	-1.459	22808
PPM1J	Protein phosphatase, Mg2+/Mn2+ dependent, 1J	4.75E-05	-1.418	333926
PRKAB2	Protein kinase, AMP-activated, beta 2 non-catalytic subunit	1.18E-03	-1.413	5565
RRAS2	Related RAS viral (r-ras) oncogene homolog 2	9.18E-07	-1.403	22800
EIF2A	Eukaryotic translation initiation factor 2A, 65kDa	1.52E-06	-1.402	83939
PPP1R12A	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	7.18E-06	-1.398	4659
HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1	4.67E-08	-1.393	7184
EIF2S2	Eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	5.66E-09	-1.365	8894

Table 4.02. Ranking of the down-regulated genes encoding member proteins of class I PI3K pathway in J3T spheres, as compared with J3T parental cells. ANOVA was performed to determine significant differences ($p < 0.005$). The table courtesy of Dr. Lisa Pang. ***Continued***

Symbol	Entrez Gene Name	p-value	Fold Change (-) indicates down-regulated	Entrez Gene ID for Human
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	1.01E-04	-1.363	1968
CHUK	Conserved helix-loop-helix ubiquitous kinase	2.07E-04	-1.349	1147
EIF3H	Eukaryotic translation initiation factor 3, subunit H	2.64E-04	-1.347	8667
PPP1R10	Protein phosphatase 1, regulatory (inhibitor) subunit 10	1.17E-03	-1.337	5514
MAPK14	Mitogen-activated protein kinase 14	1.07E-05	-1.335	1432
RPS6KA1	Ribosomal protein S6 kinase, 90kDa, polypeptide 1	5.12E-06	-1.333	6195
BMPR1B	Bone morphogenetic protein receptor, type IB	7.50E-05	-1.320	658
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5	7.45E-05	-1.289	23533
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	3.10E-06	-1.288	4893
PPP1CC	Protein phosphatase 1, catalytic subunit, gamma isozyme	1.78E-06	-1.266	5501
YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	3.85E-07	-1.255	10971
EIF4A3	Eukaryotic translation initiation factor 4A3	1.85E-04	-1.255	9775
YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	3.85E-07	-1.255	10971
PABPC1	Poly(A) binding protein, cytoplasmic 1	6.47E-04	-1.228	26986
CSNK2A1	Casein kinase 2, alpha 1 polypeptide	1.88E-05	-1.226	1457
YWHAB	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	2.84E-04	-1.223	7529
SRF	Serum response factor (c-fos serum response element-binding transcription factor)	2.82E-05	-1.219	6722
EIF3G	Eukaryotic translation initiation factor 3, subunit G	3.47E-05	-1.215	8666
GRB2	Growth factor receptor-bound protein 2	1.04E-03	-1.190	2885



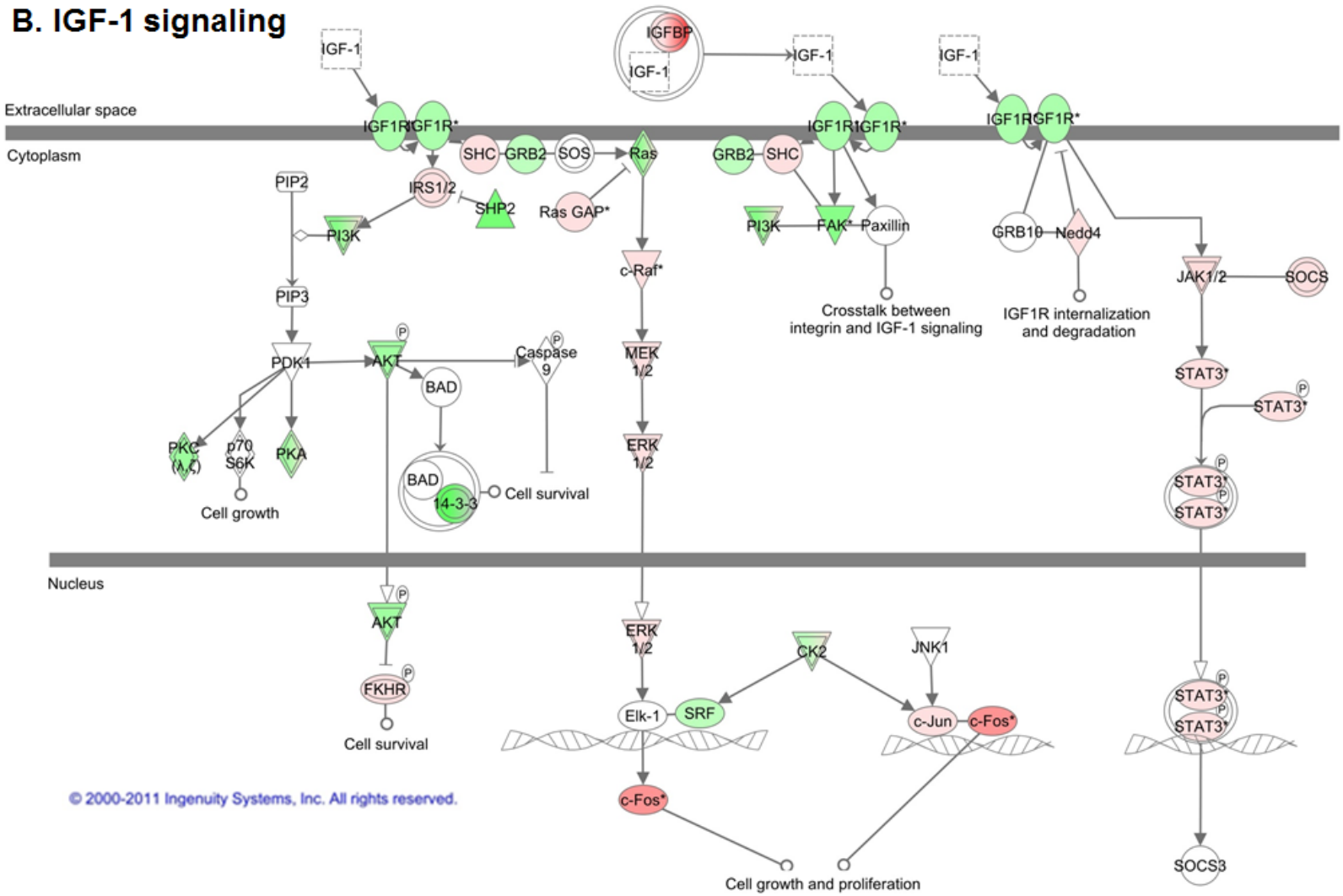

B. IGF-1 signaling
IGF-1
IGFBP
IGF-1
IGF-1
IGF-1
Extracellular space
Cytoplasm
IGF1R
IGF1R*
SHC
GRB2
SOS
Ras
GRB2
SHC
IGF1R
IGF1R*
IGF1R
IGF1R*
PIP2
IRS1/2
SHP2
Ras GAP*
PI3K
PI3K
FAK*
Paxillin
GRB10
Nedd4
JAK1/2
SOCS
c-Raf*
PIP3
Crosstalk between integrin and IGF-1 signaling
IGF1R internalization and degradation
PDK1
AKT
Caspase 9
MEK 1/2
STAT3*
STAT3*
BAD
PKC (λ,ζ)
p70 S6K
PKA
ERK 1/2
BAD
14-3-3
Cell survival
Cell growth
STAT3*
STAT3*
STAT3*
Nucleus
AKT
ERK 1/2
CK2
JNK1
FKHR
Elk-1
SRF
c-Jun
c-Fos*
STAT3*
STAT3*
Cell survival
c-Fos*
SOCS3
Cell growth and proliferation
© 2000-2011 Ingenuity Systems, Inc. All rights reserved.

Figure 4.02. Graphic representations of the networks of class I PI3K and its major downstream targets. IPA was performed to analyze microarray data sets of sphere versus parental J3T cells and the graphics of insulin receptor (IR) pathway (A), insulin-like growth factor-1 (IGF-1) pathway (B). Gene expression variation by at least 1-fold was depicted by either green (down-regulated) or red (up-regulated) colour. The darker the colour (green/red) was, the greater the value of fold-change was. ◇ represents enzyme, ◎ represents group or complex, ⬚ represents growth factor, ⋮ represents ion channel, ▽ represents kinase, ▭ represents ligand-dependent nuclear receptor, △ represents phosphatase, ⬭ represents transcription regulator, ⬡ represents translation regulator, ⬮ represents transmembrane receptor, ⏢ represents transporter, ○ represents other. The figures courtesy of Dr. Lisa Pang.

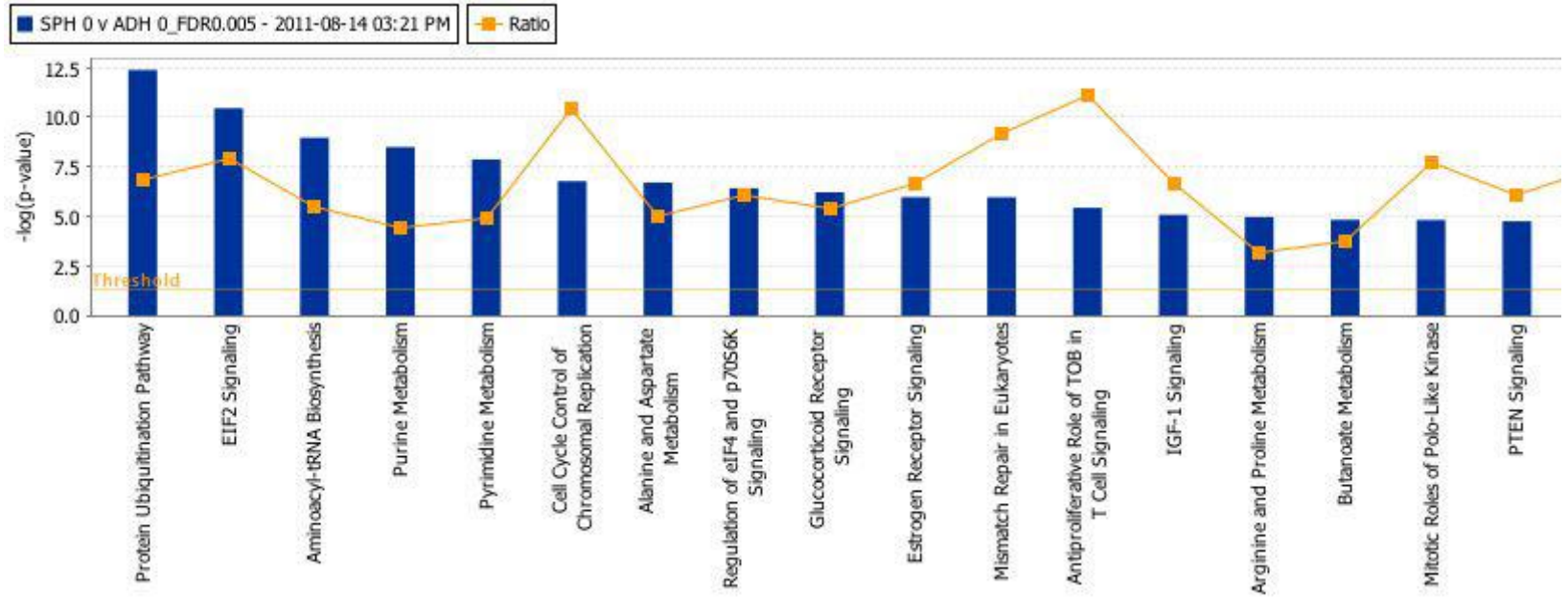


Figure 4.03. Top 17 canonical pathways relevant to the differentially expressed genes between the J3T spheres and their parental cell line, ranked by significance. IPA of the entire differentially expressed genes between the J3T spheres and their parental cells determined the degree of the relevance of each pathway to these differentially expressed genes and defined the confidence of the relevance. Yellow dot represented a ratio of the number of the differentially expressed genes pertaining to each pathway divided by the total number of genes in each pathway. Blue bar indicated the p-value of statistical significance. A high ratio represented high relevance of a given pathway to the differentially expressed genes in this pathway. A low p-value represented high confidence of this relevance. The figure courtesy of Dr. Lisa Pang.

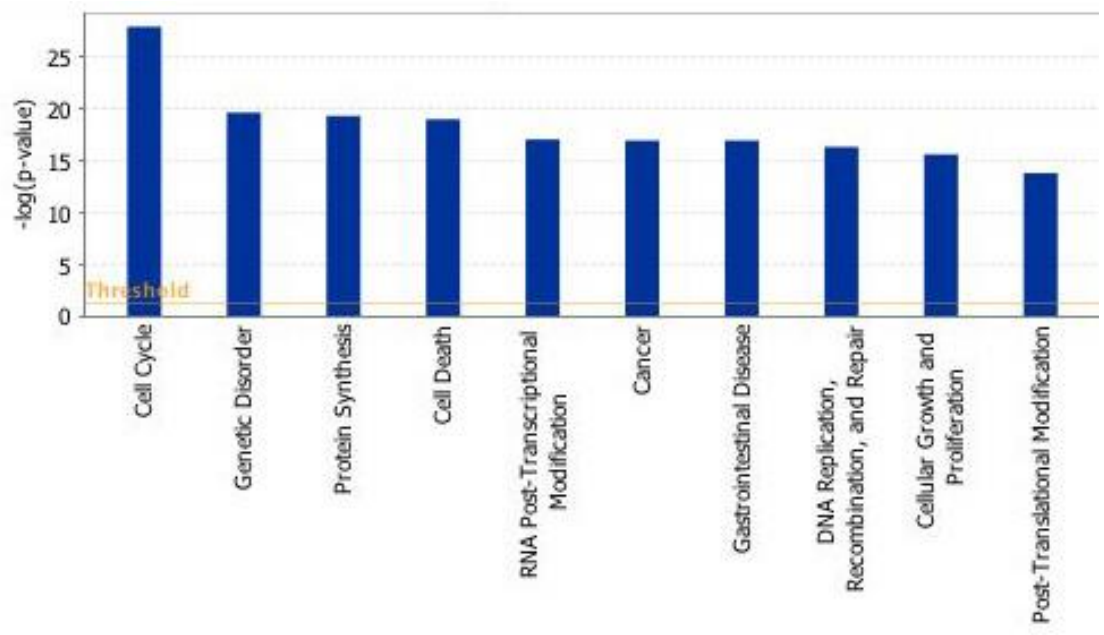


Figure 4.04. Top 10 biological functions and diseases relevant to the differentially expressed genes between the J3T spheres and their parental cell line, ranked by significance. IPA of the entire differentially expressed genes between the J3T spheres and their parental cells determined the degree of the association of a given function/disease with these differentially expressed genes. Blue bar indicated the p-value of statistical significance. A low p-value represented that a given function/disease was more significantly related to the microarray data, not because of random chance. The figure courtesy of Dr. Lisa Pang.

4.4 Discussion

To date, increasing evidence is in favor of CSC theory, implicating CSCs as a promising therapeutic target for the treatment of cancer. This study demonstrates that canine glioma J3T cells contain CSCs by using neurosphere culture system. Furthermore, cDNA microarray analysis shows that many genes are differentially expressed between J3T spheres and their parental cells. Some of these genes that have previously been described as activators of class I PI3K/Akt/mTORC1 axis pathway are CBL, CSNK2A1, CSNK2A2, DDR1, FGFR1, FGFR2, FLT1, FYN, GAB1, GHR, GRB2, HSP90B1, IGF1R, INSR, IRS1, JAK2, KDR, KRAS, MRAS, NRAS, NTRK2, NTRK3, PDGFC, PDGFRA, PDGFRB, PIK3C3, PTK2, PTPN11, RRAS2, and SHC1 (see Table 4.03) (Marshall 1995; VanderKuur *et al.* 1995; Jain *et al.* 1997; Leonard and O'Shea 1998; Wymann and Pirola 1998; Hunter *et al.* 1999; Kimmelman *et al.* 2000; Rodrigues *et al.* 2000; Sato *et al.* 2000; Herrington and Carter-Su 2001; Ravichandran 2001; Wu *et al.* 2001; Rong *et al.* 2002; Cully *et al.* 2006; Nobukuni *et al.* 2007; Radhakrishnan *et al.* 2008; Calzolari and Malatesta 2010; Guan 2010; Hanif *et al.* 2010; Lemmon and Schlessinger 2010; Saito *et al.* 2010; Castellano and Downward 2011). Among these activators, CBL, FYN, NTRK2, NTRK3 and PDGFRB are significantly up-regulated by $\geq$ 3-fold in the spheres as compared with their parental cells. Expression of FYN, NTRK2 and NTRK3 in human glioma cells and expression of PDGFR in endothelial cells in gliomas have been documented (Wang *et al.* 1998; Furnari *et al.* 2007; Yadav and Denning 2011). CBL, FYN, and NTRK2 have been reported to play a role in glioma progression. FYN up-regulates invasion and migration of glioma (Ohira *et al.* 2006). CBL promotes tumor invasion (Lee and Tsygankov 2010). Truncated NTRK2, instead of full-length NTRK2, regulates cytoskeletal arrangement and alteration of cellular morphology in glioma through its substrate RhoA (Ohira *et al.* 2006). However, not all of these activators of class I PI3K/Akt/mTOR pathway are up-regulated in the sphere cells. CSNK2A1, FGFR2, FLT1, GRB2, HSP90B1, IGF1R, KRAS, MRAS, NRAS, PTK2, PTPN11, and RRAS2 are found to be down-regulated by less than 3-fold in J3T spheres.

Some of these differentially expressed genes that have previously been described as inhibitors of class I PI3K/Akt/mTORC1 axis pathway are DDIT4, IGFBP2, IGFBP4, IGFBP6, IGFBP7, INPPL1, PLD3, PRKAR1A, PRKAR2A, PRKAB2, RASA1, SOCS2, SOCS5, STK11, THEM4 (Vogel *et al.* 1988; Damen *et al.* 1996; Oh *et al.* 1996; Rajaram *et al.* 1997; Filippa *et al.* 1999; Seki *et al.* 2002; Howe 2004; Knobbe *et al.* 2004; Reiling and Hafen 2004; Woodcock and Hughes 2004; Kario *et al.* 2005; Sofer *et al.* 2005; Hardie *et al.* 2006; Rico-Bautista *et al.* 2006; Zhang *et al.* 2009; Oakhill *et al.* 2011). Among these inhibitors, IGFBP2, DDIT4, and PLD3 are significantly up-regulated by ≥ 3-fold in the spheres as compared with their parental cells (see Table 4.03). So far, no report regarding the role of DDIT4 and PLD3 in glioma development has been published. Increased expression of IGFBP2 is commonly found in human as well as canine high-grade gliomas and is associated with poor prognosis (Becher *et al.* 2008; Lin *et al.* 2009; Stoica *et al.* 2011). Although the function of IGFBP2 protein in normal cells is to counteract IGF signaling, this protein has been reported to stimulate Akt activity in gliomas in response to platelet derived growth factor B (PDGF-B) stimulation (Rajaram *et al.* 1997; Dunlap *et al.* 2007). Previous data suggested that IGFBP2 was involved in promoting invasion and motility, and up-regulating DNA repair mechanism in high-grade gliomas (Wang *et al.* 2003; Wang *et al.* 2006; Becher *et al.* 2008). IGFBP2 was demonstrated to induce proliferation, maintain survival and drug resistance of glioma CSCs, in part through Akt signaling (Hsieh *et al.* 2010). Of these class I PI3K pathway inhibitors, three genes, which are PRKAR2A, PRKAB2, and THEM4 are found to be down-regulated in the J3T spheres.

In addition to CBL, DDIT4, FYN, IGFBP2, NTRK2, NTRK3, PDGFRB, and PLD3 genes which have previously been identified as regulators of class I PI3K/Akt/mTOR pathway and been detected to express much higher mRNA levels (> 3-fold) in the J3T spheres than their parental cells (Jain *et al.* 1997; Rajaram *et al.* 1997; Hunter *et al.* 1999; Huang and Reichardt 2003; Reiling and Hafen 2004; Sofer *et al.* 2005; Zhang *et al.* 2009; Calzolari and Malatesta 2010; Saito *et al.* 2010), there are some genes, including CTNNB1, FOS, IGF2R, PPP1R3C, and YWHAG, which are

significantly up-regulated or down-regulated (> 3-fold) in the J3T spheres. CTNNB1 encodes β-catenin, which serves as an important component of Wnt core signaling. Wnt/β-catenin signaling was reported to exert dual effects on stem cells. On one hand, this pathway acted to maintain self-renewal and pluripotent properties of ES cells and somatic SCs. On the other hand, Wnt//β-catenin signaling triggered differentiation and regulated lineage commitment of ESCs (Miki *et al.* 2011). β-catenin was reported to promote proliferation, induce chemoresistance and radioresistance, and up-regulate invasiveness and EMT in glioma (Nager *et al.* 2012; Shi *et al.* 2012). In addition, Wnt/β-catenin pathway played a key role in maintenance of glioma stem cell pool (Nager *et al.* 2012). FOS encodes c-Fos which is a subunit of activator protein-1 (AP-1) transcription factor (Hess *et al.* 2004). The levels of c-Fos are increased with tumour grading of gliomas (Yu *et al.* 1999). c-Fos was found to positively regulate proliferation of glioma cells (Koul *et al.* 2007). IGF2R encoding IGFII/mannose-6-phosphate receptor which acts to attenuate IGF-II signaling (Furstenberger and Senn 2002). Loss of IGF2R expression, as a result of chromosomal translocation, was detected in clinical specimens and cells lines of human GBM (Mulholland *et al.* 2006). PPP1R3C encodes the regulatory subunit 3C of protein phosphatase 1 (PP1), which and functions in promoting glycogen synthesis, in all kinds of cells, particularly in liver and muscle cells (Cohen 2002). So far, no report regarding the role of PPP1R3C in gliomas has been published yet. YWHAG encodes 14-3-3γ, which belongs to 14-3-3 phosphoserine/phosphothreonine-binding protein family and participate in a variety of cellular functions through binding to its protein partners (Wilker and Yaffe 2004). Previous findings showed that 14-3-3 family proteins generally promoted survival and proliferation of glioma cells (Cao *et al.* 2010).

In this study, the expression levels of four components, including p101 and p110δ subunits of class I PI3Ks, Akt1, and eIF4E, are decreased in the spheres. However, it should be borne in mind that cDNA microarray analysis shows the variation in mRNA levels but cannot show signal transduction status, such as the phosphorylation status of class I PI3K pathway. Besides, class I PI3Ks are composed of four p110

catalytic subunit isoforms (p110α, β, γ and δ) at least three regulatory subunits such as p85, p101 and p87/84 (Wymann and Pirola 1998; Suire *et al.* 2005; Voigt *et al.* 2005). There are three Akt isoforms, including Akt1, Akt2 and Akt3 (Altomare and Testa 2005). Although J3T spheres contain lower levels of transcripts encoding p110δ, p101 and Akt1, there might be a possibility that class I PI3K/Akt/mTOR signaling is activated in J3T spheres through phosphoarylation/activation of p110α, p110β, p110γ, p85 and p87/84 subunits of class PI3K, Akt2 and Akt3 and a wide array of class I PI3K and Akt substrates, such as Rac1, mTOR, GSK3 and FoxO1 (Cantley 2002). Due to this potential disvantage of cDNA microarray, techniques, such as Western Blotting and Reverse-Phase Protein Array, can be employed for comparison of phosphorylation status of class I PI3K/Akt/mTOR signaling proteins in the J3T spheres with that in the J3T parental cells (Zhou *et al.* 2007). Hence, it cannot be ruled out the possibility that class I PI3K/Akt/mTOR is activated through phosphorylation of the components whose expression levels are not decreased.

In J3T spheres, 22 transcripts encoding proteins involved in regulation of translation initiation are down-regulated (Table 4.02). Of these translation initiation factors, EIF2S1 and EIF3B have been reported to participate in glioma progression by up-regulating cell cycle progression (by EIF2S1 and EIF3B) and survival (by EIF3B) of high-grade glioma cells (Kambara *et al.* 2005; Liang *et al.* 2012). 5 transcripts, including EIF1, EIF2C1, EIF2C3, PAIP1 and PAIP2, are up-regulated. EIF1 and PAIP1 express proteins which enhance translation initiation whereas EIF2C1, EIF2C3 and PAIP2 act to inhibit mRNA translation (Khaleghpour *et al.* 2001; Doi *et al.* 2003; Preiss and Hentze 2003; Meister *et al.* 2004). There is no published data about the 5 up-regulated genes. Moreover, the mRNA levels of EIF4E, which encodes the key component of mTOR pathway and trigger cap-dependent mRNA translation, is found being down-regulated (Huang and Houghton 2003). Taken together, it is suggested that translation initiation mechanism and cap-dependent mRNA translation are down-regulated in the J3T spheres.

In this study, several class I PI3K/Akt/mTOR pathway-related transcripts which are differentially expressed in J3T spheres have previously been identified to be up-regulated in human glioma stem cells (GSCs) as well. The class I PI3K pathway-associated transcripts, including anti-apoptotic factors BCL2 and BCL2L1, IGFBP2, integrin receptor ITGB1, and PRKCD encoding protein kinase C δ (PKCδ), are increased in both canine J3T spheres and human GSCs (Liu *et al.* 2006; Gunther *et al.* 2008; Hsieh *et al.* 2010; Lamszus and Gunther 2010; Lathia *et al.* 2010). IGFBP2 was demonstrated to induce proliferation, maintain survival and drug resistance of glioma CSCs, in part through Akt signaling (Hsieh *et al.* 2010). ITGB1 was reported to up-regulate self-renewal, proliferation and tumourigenic capabilities of GSCs (Lathia *et al.* 2010). PRKCD was found to up-regulate self-renewal capability of glioma upon radiotherapy, suggesting that PRKCD might play a role in radioresistant mechanism (Kim *et al.* 2011). In addition, some transcripts, including AKT1, BMPR1B, CTNNB1, GSK3B, JAK2, PPP2CB, PIK3CD, PIK3R5, RAC1, STAT3, and TGFBR2, which are identified to be expressed differentially between the spheres and parental cells have previously been found to be involved in regulation of many processes of human GSCs (see Table 4.03) (Lee *et al.* 2008; Korur *et al.* 2009; Howard *et al.* 2010; Hsieh *et al.* 2010; Lamszus and Gunther 2010; Lathia *et al.* 2010; Liu *et al.* 2010; Kim *et al.* 2011; Yoon *et al.* 2011; Nager *et al.* 2012; Sai *et al.* 2012). Previous studies showed that class I PI3K, Jak2/Stat3, TGFβ and Wnt/β-catenin pathways and Rac1 up-regulated stem cell properties including self-renewal, multipotency and proliferation, and malignant behaviours, including survival, invasion, migration, immune suppression, chemoresistance and radioresistance (Lamszus and Gunther 2010; Liu *et al.* 2010; Yoon *et al.* 2011; Miyazono *et al.* 2012; Nager *et al.* 2012; Sai *et al.* 2012). Conversely, BMP signaling and protein phosphatase 2A (PP2A) acted to induce differentiation and/or inhibit survival of GSCs (Lee *et al.* 2008; Lu *et al.* 2010). There is conflicting data regarding the role of GSK-3β in GSCs. There is conflicting data regarding the role of GSK-3β in glioblastoma stem cells. On one hand, active EGFR/Akt signaling-mediated inhibitory phosphorylation of GSK-3β was found to promote proliferation of neurosphere cells (Howard *et al.* 2010). On the other hand, down-regulation of GSK3B

expression or GSK-3β activity reduced GBM stem cell pool and induced differentiation and apoptosis (Korur *et al.* 2009). The present study displays increased CTNNB1 (β-catenin), JAK2, STAT3, PPP2CB (catalytic subunit-beta isoform of PP2A), and RAC1 transcripts, but decreased BMPR1B (type I BMP receptor), TGFBR2 (TGFβ receptor type II), PIK3CD, PIK3R5 and AKT1 transcripts. This suggests that Wnt/β-catenin and Jak2/Stat3 pathways, PP2A, GSK3B and Rac1 activity are up-regulated whereas BMP, TGFβ and class I PI3K pathways are down-regulated in the J3T spheres.

Among these differentially expressed genes between the J3T spheres and parental cells, many of them were previously identified to regulate glioma development, including cell cycle progression, survival, invasion, migration, adhesion, chemoresistance, radioresistance, angiogenesis and CSC-associated properties. The genes which have previously been identified to promote cell cycle progression of glioma cells are BCL2, CTNNB1, EIF2S1, EIF3B, EIF4E, FGFR1, FOS, GSK3B, HSP90B1, IGF1R, IGFBP10, INSR, JAK2, PTK2, MAP2K1, MAPK1, PRKCI, STAT3, YWHAB, YWHAG and YWHAQ (Glick *et al.* 1989; Yamada *et al.* 1998; Natarajan *et al.* 2003; Kambara *et al.* 2005; Pelloski *et al.* 2006; Koul *et al.* 2007; Sciaccaluga *et al.* 2007; Messaoudi *et al.* 2008; Dubois *et al.* 2009; Hargrave 2009; Miyashita *et al.* 2009; Cao *et al.* 2010; Goodwin *et al.* 2010; Hagerstrand *et al.* 2010; Liu *et al.* 2010; Rouschop *et al.* ; Desai *et al.* 2012; Kouri *et al.* 2012; Liang *et al.* 2012; Nager *et al.* 2012). In addition, since PKA negatively regulates cellular proliferation in gliomas, PRKAR1A and PRKAR2A, both of which are inhibitors of PKA, are suggested to up-regulate proliferation of tumour cells (Chen *et al.* 1998). The role of FGFR2 in up-regulating cellular proliferation of gliomas was cell line-specific (Yamada *et al.* 1999; Auguste *et al.* 2001). The cDNA microarray data show that BCL2, CTNNB1, FGFR1, FOS, INSR, JAK2, GSK3B, MAP2K1, PRKAR1A, MAPK1, and IGFBP10 STAT3 transcripts are increased. In contrast, EIF2S1, EIF3B IGF1R, FGFR2, PTK2, HSP90B1, EIF4E, PRKCI, PRKAR2A, YWHAB, YWHAG, and YWHAQ are down-regulated in the J3T spheres. Previous studies showed that INPPL1, MAPK14, RAF1, RND3, STK11, and TGFBR2 inhibited cell cycle progression (Taylor *et al.* 2000; Fanton *et al.* 2001; Wick *et al.* 2006; Poch *et al.* 2007; Yao *et al.* 2008; Godlewski *et al.* 2010). The J3T spheres

are found to contain higher mRNA levels of INPPL1, RAF1 and RND3 but lower mRNA levels of STK11, TGFBR2 and MAPK14 than the J3T parental cells. Taken together, it is suggested that up-regulation of the genes encoding positive regulators of cell cycle progression such as CTNNB1 and down-regulation of the genes encoding inhibitors of cell cycle transition such as EIF2S1 may be involved in up-regulating the growth and proliferation of the J3T spheres. On the contrary, down-regulation of activators such as INPPL1 of cell cycle transition and up-regulation of cell cycle inhibitors such as STK11 are implicated to down-regulate expansion ability of the tumour spheres.

The genes which have previously been identified to promote survival of glioma cells are BCL2, BCL2L1, CSNK2A1, CSNK2A2, EIF3B, MAP2K1, MAPK1, MCL1, and PPP1CC GSK3B, HSP90B1, IGFBP2, PRKAB2, PRKCI, PTK2, STK11, STAT3, YWHAB, YWHAG, YWHAQ (see Table 4.03) (Natarajan *et al.* 2003; Pelloski *et al.* 2006; Weiler *et al.* 2006; Becher *et al.* 2008; Messaoudi *et al.* 2008; Hargrave 2009; Harhaji-Trajkovic *et al.* 2009; Miyashita *et al.* 2009; Cao *et al.* 2010; Godlewski *et al.* 2010; Liu *et al.* 2010; Olsen *et al.* 2010; Sheng *et al.* 2010; Desai *et al.* 2011; Kelsall *et al.* 2011; Kouri *et al.* 2012; Liang *et al.* 2012). In addition, since PKA induce apoptosis in gliomas, PRKAR1A and PRKAR2A, both of which are inhibitors of PKA, are suggested to promote survival of tumour cells (Chen *et al.* 1998; Howe 2004). The BCL2, BCL2L1, CSNK2A2, MAP2K1, MAPK1, MCL1, GSK3B, IGFBP2 and STAT3 transcripts are increased in the J3T spheres, as compared with their parental cells. Conversely, CSNK2A1, EIF3B, PPP1CC, HSP90B1, PRKAB2, PRKCI, PTK2, STK11, YWHAB, YWHAG, and YWHAQ transcripts are decreased in the J3T spheres. MAPK14, PRKCD and RND3 were previously reported to induce apoptosis in glioma cells. The J3T spheres are detected to contain higher mRNA levels of PRKCD and RND3 but lower mRNA levels of MAPK14, as compared with the J3T parental cells. Taken together, it is suggested that up-regulation of the genes encoding anti-apoptotic factors such as BCL2 and down-regulation of pro-apoptotic factor MAPK2 may contribute to promote survival of the J3T sphere cells. By contrast, down-regulation of

anti-apoptotic factors such as EIF3B and up-regulation of pro-apoptotic factors such as RND3 may induce apoptosis of the J3T spheres.

The genes which have previously been identified to enhance invasion of glioma cells are ACLY, BCL2L1, CBL, CHUK, DDR1, FYN, HSP90B1, IGFBP2, JAK2, PIK3CD, PRKCI, PTK2, RAC1, CTNNB1, MAPK1, MAPK14, RAF1, PRKCD, IGFBP10, RHOC and TGFBR2 (Natarajan *et al.* 2003; Wang *et al.* 2003; Salhia *et al.* 2005; Nakada *et al.* 2006; Ram *et al.* 2006; Weiler *et al.* 2006; Wick *et al.* 2006; Demuth *et al.* 2007; Messaoudi *et al.* 2008; Sasayama *et al.* 2009; Young *et al.* 2009; Baldwin *et al.* 2010; Beckner *et al.* 2010; Lee and Tsygankov 2010; Sarkar and Yong 2010; Das *et al.* 2011; Senft *et al.* 2011; Yadav and Denning 2011; Luk *et al.* 2012; Nager *et al.* 2012; Shi *et al.* 2012; Zhang *et al.* 2012). In this study, up-regulation of ACLY, BCL2L1, CBL, DDR1, FYN, IGFBP2, JAK2, PRKCI, RAC1, CTNNB1, MAPK1, RAF1, PRKCD, IGFBP10, and RHOC genes in the J3T spheres when compared to their parental cells suggests that these highly expressed genes may increase invasive behavior for the J3T spheres. On the contrary, down-regulation of the genes of CHUK, HSP90B1, PIK3CD, PTK2, MAPK14, and TGFBR2 in the J3T spheres suggests that regulation of the invasive phenotype for the J3T spheres is irrelevant to these genes.

The genes which have previously been identified to promote migration or metastasis of glioma cells are ACLY, CHUK, CTNNB1, FYN, HSP90B1, IGFBP2, IGFBP10, INPPL1, ITGA5, JAK2, PIK3CD, PRKAB2, PTK2, RAC1, STK11, MAPK14, MAPK1, SRF and TGFBR2 (Damen *et al.* 1996; Natarajan *et al.* 2003; Salhia *et al.* 2005; Hardie *et al.* 2006; Song and Moon 2006; Wang *et al.* 2006; Wick *et al.* 2006; Demuth *et al.* 2007; Messaoudi *et al.* 2008; Beckner *et al.* 2010; Goodwin *et al.* 2010; Tabatabai *et al.* 2010; Oakhill *et al.* 2011; Senft *et al.* 2011; Yadav and Denning 2011; Ziv-Av *et al.* 2011; Luk *et al.* 2012; Nager *et al.* 2012; Shi *et al.* 2012; Zhang *et al.* 2012). Among these genes, ACLY, CTNNB1, FYN, IGFBP2, IGFBP10, INPPL1, ITGA5, JAK2, RAC1, STK11 and MAPK1 are up-regulated in the J3T spheres when compared to their parental cells, whereas CHUK, HSP90B1, PIK3CD, PRKAB2, PTK2,

MAPK14, SRF and TGFBR2 are down-regulated in the sphere cells. Besides, two genes, RHOA and PRKCD, which have previously been identified to attenuate migration of glioma cells are found to be up-regulated in the J3T spheres (Cachero *et al.* 1998; Ziv-Av *et al.* 2011). Taken together, it is suggested that up-regulation of the genes, such as ACLY and CTNNB1, which are involved in inducing migration may play a role to up-regulate the migration behavior for the J3T spheres. Conversely, it is suggested that either down-regulation of the genes (e.g. CHUK and HSP90B1) involved in promoting migration, or up-regulation of the genes (e.g.RHOA and PRKCD) associated with blockade of migration may weaken the migration/metastasis capability of the J3T spheres.

In addition to the genes which are related to invasion, migration, and metastasis, certain genes are involved in cytoskeletal arrangement and alteration of cell morphology. Expression of truncated NTRK2, instead of full-length NTRK2, was reported to regulate RhoA-modulated cell morphology and cytoskeletal rearrangement in rat C6 glioma cell line in response to brain-derived neurotrophic factor (BDNF) (Ohira *et al.* 2006). RND3, which encoded RhoE, was thought of as tumour suppressor in glioblastoma by not only impairment of cytoskeletal arrangement, but also inhibition of survival and growth (Poch *et al.* 2007).

The genes which have previously been identified to promote angiogenesis or vascular formation within gliomas are FGFR1, FGFR2, FLT1, HSP90B1, STAT3 and ITGA5 (Auguste *et al.* 2001; Xiang *et al.* 2001; Liu *et al.* 2010; Tabatabai *et al.* 2010). In this study, the transcriptional levels of FGFR1, STAT3 and ITGA5 are increased in the J3T spheres whereas those of FGFR2, FLT1 and HSP90B1 are decreased. Besides, the genes which have previously been identified to inhibit angiogenesis of glioma cells are IGFBP4 and MAPK14 (Moreno *et al.* 2006; Yoshino *et al.* 2006). The J3T spheres express higher levels of IGFBP4 transcript but lower levels of MAPK14 transcript than the J3T parental cells. Collectively, it is suggested that up-regulation of these genes (e.g. FGFR1) which positively regulate angiogenesis in the J3T spheres or down-regulation of MAPK14 which functions in blockade of angiogenesis may contribute to blood vessel

formation and sufficient oxygen for glioma development. Conversely, it is suggested that down-regulation of the genes (e.g. FGFR2) which positively regulate angiogenesis or up-regulation of IGFBP4 which acts to inhibit angiogenesis may serve as tumour suppressors which antagonize glioma progression.

In this study, the class I PI3K signaling network graphics which are generated by using IPA suggests up-regulation of IR signaling in the J3T spheres, based on increased transcriptional levels of the genes encoding IR and many of its adaptors and decreased transcription of the gene encoding IGF-1R. IR is mainly activated by insulin and plays a critical role in regulation of carbohydrate metabolism. IGF-1R is mainly activated by IGF-1 and IGF-2 and this receptor is involved in regulation of cell growth. Under certain circumstance, IR can be activated by IGF-2 and IGF-1R can be activated by insulin when IR isoform A dimerizes with IGF-1R (Pollak 2012). Despite no report concerning the roles of IR and IGF-1R in glioma stem cells, accumulating evidence suggests that both receptors regulate neurogenesis by controlling self-renewal and differentiation of NSCs. IGF-1 has been found to promote cycling (proliferation) of NSCs, followed by initiate differentiation of NSC into more mature neural cells. Insulin has been speculated to maintain survival of NSC by regulating nutrient metabolism in NSC (Rafalski and Brunet 2011). Taken together, increased transcription of INSR (encoding IR) and decreased transcription of IGF1R (encoding IGF-1R) in the J3T spheres indicate that IR expression, instead of IGF-1R, may play a role in regulation of CSC biology of the J3T cells, presumably through regulation of nutrient metabolism in the spheres.

The IPA-generated signaling network graphics suggest that the J3T spheres contain hypo-activities of class I PI3K/Akt/mTOR pathway and eIF4E-modulated cap-dependent translation but hyper-activities of Raf1/Mek1/Erk and Jak2/Stat3 pathways. Besides, it appears that PTEN activity is enhanced in the spheres, as evidenced by decreased CK2 transcript, a PTEN negative regulator, and increased MAGI transcripts (Simpson and Parsons 2001; Hanif *et al.* 2010). However, previous manuscripts often addressed the important role of class I PI3K/Akt pathway, particularly Akt activity, in NSC and GSC biology (Eyler *et al.* 2008; Bleau *et al.* 2009; Rafalski and Brunet 2011).

For example, an *in vitro* study showed that increased Akt activity in neural progenitors isolated from adult murine brain, by introducing overexpressed Akt to these progenitors, enhanced proliferation but attenuated differentiation capabilities of these neural progenitors (Peltier *et al.* 2007). Similar results obtained from another research group showed that murine neurospheres with PTEN loss increased proliferation of NSCs but did not affect the differentiation capability of NSCs. Furthermore, these PTEN-null NSCs produced enlarged but aberrant brain in mouse model, due to up-regulating the growth, proliferation and survival of NSCs (Groszer *et al.* 2001). Generally, accumulating evidence suggests that up-regulation of Akt signaling in neural SCs/progenitors from either adult or postnatal mouse brains up-regulates self-renewal, proliferation and survival of these neural primitive cells but does not necessarily attenuate differentiation capability of these cells (Groszer *et al.* 2001; Peltier *et al.* 2007). In addition, some Akt substrates, mTORC1 and FoxO transcription factors, were found to regulate cellular processes of NSCs. These Akt substrates were modulated not only by active Akt in response to insulin/insulin-like growth factor (IGF) stimulation but also by other upstream inputs such as Notch and CD95 stimuli. Whilst mTORC1 was found to play a role in neurogenesis through up-regulation of translation, survival and differentiation, FoxO transcription factors, such as FoxO3, were more likely to maintain NSCs at primitive state and preserved SC properties such as self-renewal and multipotency, prevented quiescent NSCs from entry to cell cycling, inhibited differentiation, and protected cells from oxidative resistance (Rafalski and Brunet 2011). As to the role of class I PI3K/Akt/mTOR pathway in GSC biology, evidence showed the important roles of both Akt and mTOR in regulation of cellular processes of GSCs (Eyler *et al.* 2008; Bleau *et al.* 2009; Jhanwar-Uniyal *et al.* 2011). A study showed that Akt increased the fraction of stem cell phenotype in both human and mouse gliomas via mTOR-independent downstream signaling (Bleau *et al.* 2009). Another study showed that CD133+ cells (enriched for putative CSCs) which were isolated from human glioblastomas were more sensitive to Akt inhibition than their non-CSC counterparts (CD133- tumour cells), as evidenced by remarkable decline in cell viability and attenuation of migration and invasion capabilities in CD133+ glioblastoma cells *in vitro*.

Moreover, mouse model transplanted with CD133+ human glioblastoma cells in which Akt activity was inhibited was observed to have prolonged survival time (Eyler *et al.* 2008). It was suggested that mTOR, particularly mTORC1, might exert dual effects on human GSC biology. On one hand, down-regulation of mTORC1 signaling by Rapamycin was observed to reduce neurosphere colonies which were derived from human GBM cells, suggesting the involvement of mTORC1 in positively regulating self-renewal. On the other hand, mTOR or MAPK inactivation was found to induce nestin (a SC marker) expression in the human GBM cells, indicating that these pathways might participate in reversion of differentiation process and maintenance of GSC multipotency (Jhanwar-Uniyal *et al.* 2011). Taken together, previous data obtained from NSCs and GSCs in human or mouse showed that Akt activity was involved in up-regulating proliferation, self-renewal and survival of NSC and GSCs, and increased GSC malignant behaviors, including multidrug-resistant mechanism (ABCG2 activity), invasion and migration (Groszer *et al.* 2001; Peltier *et al.* 2007; Eyler *et al.* 2008; Bleau *et al.* 2009). In contrast to previous findings, it appears that the class I PI3K/Akt/mTOR pathway is dispensable for cellular activities of canine GSCs (the J3T spheres).

It is observed that transcription of the genes encoding Raf1, Mek1, and Erk2 are slightly up-regulated by 1.3~1.7 fold in the J3T spheres when compared to their parental cells. Previous reports described that Mek/Erk MAPK pathway was involved in regulation of several processes of NSCs, including self-renewal, multipotency, differentiation and survival (Rueda *et al.* 2002; Hao *et al.* 2004; Imamura *et al.* 2008; Ohtsuka *et al.* 2009; Wang *et al.* 2009; Phoenix and Temple 2010; Gan *et al.* 2011). Erk MAPK pathway was found to up-regulate survival of murine and rat NSC following stimulation of apolipoprotein E or a mood stabilizer Valproate (Hao *et al.* 2004; Gan *et al.* 2011). The Erk pathway-modulated NSC survival was, in part, through up-regulation of survival genes such as BCL2 (Hao *et al.* 2004). However, previous studies about elucidating the role of this pathway in self-renewal, multipotency, and differentiation of NSCs, were controversial. Many studies addressed that Raf/Mek/Erk MAPK pathway contributed to neurogenesis, through inducing proliferation and subsequent

differentiation of NSCs, upon stimulation of neurotrophins such as nerve growth factor (NGF) and neurotrophin 3 (NT3), Valproate, and BMP4 (Huang and Reichardt 2003; Hao *et al.* 2004; Moon *et al.* 2009). Moreover, down-regulation of Erk signaling by endocannabinoids was found to suppress differentiation of neural primitive cells (Rueda *et al.* 2002). However, some studies showed the opposite effects of Erk pathway, particularly Erk2 activity, on NSC/neural progenitors (Imamura *et al.* 2008; Samuels *et al.* 2008; Wang *et al.* 2009; Phoenix and Temple 2010). For instance, a study in mouse model showed that Erk2-null NSCs attenuated cell proliferation. Moreover, Erk2-null neural progenitors resulted in not only cell cycle arrest but also disruption of self-renewal, multipotency and induction of apoptosis (Imamura *et al.* 2008). Similar effects on NSCs with Erk2 loss were observed by another research group (Samuels *et al.* 2008). U0126-modulated Erk1/2 inhibition was observed to inhibit proliferation and initiate differentiation of NSCs (Wang *et al.* 2009). Inhibition of Spred1, acting as negative regulator of Erk pathway, was observed to up-regulate self-renewal and proliferation of NSCs through the release of Erk MAPK pathway (Phoenix and Temple 2010). With regard to the role of Raf/Mek/Erk pathway in GSCs, previous data showed that this pathway, particularly Erk1/2 pathway, reduced the expression levels of nestin and induced differentiation of GSCs. It was suggested that Erk1/2 pathway was responsible for down-regulation of pluripotency and up-regulation of differentiation in human GSCs (Karsy *et al.* 2010; Jhanwar-Uniyal *et al.* 2011). Collectively, it appears that the role of Raf1/Mek1/Erk2 in canine GSCs is in contrast to previous findings which revealed that this pathway promoted differentiation of human GSCs. It is suggested that the Raf1/Mek1/Erk2 pathway may be involved in maintenance of undifferentiated state and viability of canine GSCs (the J3T spheres).

Previous manuscripts showed that Jak/Stat pathway was essential for maintaining self-renewal in murine ESCs, but was dispensable for maintaining monkey ESCs at undifferentiated state (Matsuda *et al.* 1999; Burdon *et al.* 2002; Sumi *et al.* 2004). For NSCs, this pathway was essential for cell fate determination, preferentially directing NSCs to astroglial differentiation rather than neuronal differentiation (Bonni *et al.* 1997;

Rajan and McKay 1998; Gu *et al.* 2005). In contrast to the effects of Jak2/Stat3 pathway on NSCs, accumulating evidence showed that this pathway exerted opposite effects on GSCs. For instance, a previous report showed that loss of Stat3 expression prevented propagation and reduced expression of NSC markers, suggesting that this pathway was critical for sustaining self-renewal and multipotency of GSCs (Sherry *et al.* 2009). Other effects, including maintenance of GSC survival and suppression of immune response, on GSCs were documented (Liu *et al.* 2010; Sai *et al.* 2012). Collectively, the current findings which reveal up-regulation of Jak2/Stat3 pathway in the J3T spheres are consistent with previous findings on human GSCs. This suggests that the effects, including maintenance of self-renewal, multipotency and survival, and immunosuppression, of the Jak2/Stat3 pathway on canine GSCs may be similar to those on human GSCs (Sherry *et al.* 2009; Sai *et al.* 2012).

The current data show that the translation machinery in the J3T spheres is down-regulated, as evidenced by decreased mRNA levels of many genes encoding translation initiation factors and translation initiation enhancers such as eIF4E and poly(A) binding protein, cytoplasmic 1 (PABP1), and increased mRNA levels of genes encoding translation initiation suppressors such as eIF2C, isoform1 and poly(A) binding protein interacting protein 2 (PAIP-2) (Khaleghpour *et al.* 2001; Doi *et al.* 2003; Mamane *et al.* 2004). One possible explanation for the down-regulated translation machinery in the J3T spheres is that these canine GSCs tested in the current study may be at the resting/quiescent state. This explanation is based on the reason that normal SCs and CSCs can be either at quiescent state which may account for drug and radiation resistant mechanism, or at cycling state for expanding stem cell pool (Roth and Fodde 2011). Besides, the J3T neurosphere colonies in serum-free culture propagated slower and more difficult than their parental cells in serum-containing culture (Reynolds and Weiss 1992).

In the current study, the results of IPA canonical pathway analysis indicate that the pathways involved in regulation of cell cycle and protein synthesis are the most significantly relevant to the entire differentially expressed genes between the J3T sphere

and their parental cells. IGF-1R, PTEN, and the regulation of p70S6K and eIF4E pathways, which are known for regulation of protein synthesis or cell growth, are found to be highly relevant to the genetic expression variation between the two cell groups. On the contrary, the Erk MAPK and Jak/Stat pathways are less relevant to these altered gene expression, as compared with IGF-1R, PTEN, and eIF4E pathways. In line with this, the results of IPA functional analysis address that the genetic expression patterns of cell cycle and protein synthesis are greatly altered between the two cell groups. Taken together, this highlights that cell cycle and protein synthesis are the most altered cellular functions in the J3T spheres.

In conclusion, the putative stem cells have been shown to be successfully isolated from canine glioma J3T cells by Sphere Formation Assay. Further, by using cDNA microarray analysis, the genes which are differentially expressed between the J3T spheres and their parental cells have been successfully identified. IPA of these differentially expressed genes further classified these genes into each canonical pathway. Of the genes belonging to members of class I PI3K signaling network, several genes including IGFBP2, FYN, NTRK2, NTRK3, FOS, PDGFRB, CBL, IGF2R, CTNNB1, PLD3 and YWHAG are significantly up-regulated or down-regulated in the J3T spheres and have previously been related to gliomagenesis and glioma progression in human/mouse/rat species. Some genes, including DDIT4, PLD3 and PPP1R3C, which are highly up-regulated in the J3T spheres are, at the first time, found to be related to glioma development. Future work will validate the current microarray data and subsequently investigate the roles of the genes whose expression levels are significantly different between the two cell groups. In order to validate microarray data, techniques such as Western blotting can be employed to investigate if significant differences in mRNA expression correlate with changes in protein production. Additionally, Northern blot analysis can be used to confirm that singinficant fold changes identified in microarray data sets correlate with measurable changes in mRNA expression in the cell lines (Al Moustafa *et al.* 2002; Chuaqui *et al.* 2002). With regard to the investigation of the roles of the genes with highly differential expression ratios, there are some

approaches for such investigation. One of these approaches is to alter the expression levels of these genes in the spheres by transfection of siRNA (to down-regulate the expression of a specific gene), or delivery of a constitutively activated gene of interest into the sphere cells (for gene overexpression). Subsequently, functional assays such as cell viability and apoptotic assays, are performed on both of the spheres with genetic modifications by siRNA or genetic overexpression, and the control spheres. Thus, the results of these functional assays may provide some clues regarding the effects exerted by the genes of interest on the J3T spheres.

IPA of the current microarray data indicate that the expression patterns of canonical pathways involved in the regulation of cell cycle and protein synthesis are remarkably altered in the J3T spheres. Since the majority of the transcripts encoding eukaryotic initiation factors that initiate mRNA translation are decreased and the transcripts that encoding the factors against translation initiation are increased, it is conceivable that down-regulation of translation initiation causes fewer mRNAs being placed on the ribosomes and being subsequently translated to polypeptides (Preiss and Hentze 2003). As a result, the protein synthesis is down-regulated in the J3T spheres. From the current microarray data, it is suggested that the J3T sphere cells display reduced activities of class I PI3K/Akt/mTOR and IGF-1R pathways and increased activities of Raf/Mek/Erk and Jak/Stat pathways, when compared to their parental cells. However, the cDNA microarray data fail to distinguish whether these pathways are in the active or inactive state. Further investigation of the activities, such as phosphorylation status, of these pathways by Western Blot and Reverse-Phase Protein Array can corroborate the current findings from the microarray data.

Chapter 5: General discussion

This study demonstrates that class I PI3K/Akt pathway plays an important role in the maintenance of viability and survival of the cell lines derived from a variety of canine tumours, as evidenced by the effective inhibition of the survival and viability of these canine cells by the inhibitors specifically targeting components of this pathway. However, while these class I PI3K/Akt/mTOR pathway inhibitors show cytotoxic or cytostatic effects on these cancer cells, these inhibitors are found to induce hyper-phosphorylation of eIF4E. Slight hyper-phosphorylation of Akt is occasionally observed in canine REM and J3T cells in the presence of Rapamycin. This suggests that these inhibitors potentially trigger Erk and/or p38MAPK survival pathways which are activators of eIF4E and release p70S6K/IRS1/PI3K/Akt feedback loop (Wang *et al.* 2007; Bianchini *et al.* 2008). To overcome the resistance induced by these class I PI3K/Akt/mTOR pathway inhibitors and simultaneously avoid the issue of maximum tolerated dose, future study can investigate the effects of the combination of inhibitors which target both class I PI3K/Akt and Mek/Erk pathways. In addition, to warrant that these pathway inhibitors can be safely administered to canine patients, future study should investigate the cytotoxic effects of these inhibitors, especially ZSTK474 and KP372-1, on normal canine cells and the side effects/toxicities in normal dogs.

It is observed that the Western Blot profiles of the five canine cell lines before and after the treatment of these PI3K pathway inhibitors are slightly different. This suggests that the molecular alterations and cellular contexts vary from one cell line to another line. Future work may investigate the genetic and epigenetic changes and the expression levels of the genes encoding components of the class I PI3K signaling network by utilizing molecular techniques such as polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) analysis, methylated DNA immunoprecipitation (MeDIP), and Western Blotting (Hayashi 1991; Mohn *et al.* 2009).

Previous manuscripts demonstrated that Rapamycin at nanomolar concentrations specifically inhibited mTORC1 and exerted cytostatic effects on most cancer cell lines,

whereas this compound at micromolar concentrations inhibited both mTORC1 and mTORC2 and exerted cytotoxic effects on cells (Shor *et al.* 2008; Chen *et al.* 2010). Consistent with previous findings, the cell viability results show that the lower micromolar concentrations (20-40 μM) of Rapamycin drive all of the canine cell lines to death. This suggests that mTORC2 may play a role in maintaining cell viability in these canine cells. To investigate the role of mTORC2 and identify its downstream pathways or targets in these canine cells, one approach is to silence the genes which encode components of mTORC2, followed by compare the activated state of the cells with decreased mTORC2 activity with that of the control cells by using Western Blotting and Reverse-Phase Protein Array. Unraveling the role of mTORC2 in these canine cells may provide a better strategy to effectively treat canine cancer.

It is observed that chronic treatment of cells with KP372-1 often causes protein loss. Since it was reported that the protein leakage and DNA fragmentation often occurred in the apoptotic cells at the secondary necrosis stage when these apoptotic cells were cultured in the medium in the absence of phagocytes (Kelly *et al.* 2003; Krysko *et al.* 2008), it is speculated that the same scenario tends to occur in these KP372-1-treated cells. To know whether protein loss in the KP372-1-treated cells is due to protein leakage, collection of both cell lysates and growth medium in the KP372-1 treatment group are required for protein quantification and Western Blot analysis.

In this study, Doxorubicin combined with the class I PI3K/Akt/mTOR pathway inhibitors do not exert synergistic or additive effects on most canine cell lines. One possible explanation for such results can be attributed to the actions of these inhibitors on G1/S cell cycle arrest, which prevents Doxorubicin from binding to proliferative cancer cells (Opel *et al.* 2008; McDonald *et al.* 2010; Bender *et al.* 2011). To improve the efficacy of Doxorubicin combined with PI3K pathway inhibitors, cells could be pretreated with Doxorubicin for 24 hours, and then concomitantly treated with Doxorubicin and the PI3K pathway inhibitors for a further ≥ 48 hours. This approach might prevent cells from accumulating at G0/G1 phase and allow Doxorubicin to interact with more cells at S phase (McDonald *et al.* 2010).

To investigate whether these class I PI3K pathway inhibitors induce autophagic resistance, future work may employ techniques such as transmission electron microscopy analysis, acridine orange staining, and autofluorescent monodansylcadaverine staining, to detect autophagic vacuoles in cells (Takeuchi *et al.* 2005; Chiarini *et al.* 2010). In addition, to overcome the drug-induced autophagic resistance, concomitant treatment of these canine cells with the class I PI3K pathway inhibitors and an autophagic inhibitor such as Chloroquine may enhance the anti-cancer activity of these class I PI3K pathway inhibitors (Fan *et al.* 2010)

The study has successfully isolated the putative stem cells from canine glioma J3T cells by sphere formation assay. Further, several genes have been identified by using cDNA microarray analysis and have been implicated to be highly associated with the phenotype of the J3T spheres, due to their much higher or lower expression levels detected in the spheres than their parental cells To investigate the roles of these differentially expressed genes, particularly IGFBP2, FYN, DDIT4, PPP1R3C, NTRK2, FOS, PDGFRB, CBL, IGF2R, and CTNNB1, microarray data must be validated, for example, by using Western blotting or Northern blotting to check whether there is difference between protein and mRNA expression in both of the J3T spheres and their parental cells (Al Moustafa *et al.* 2002; Chuaqui *et al.* 2002). After the validation of the microarray data, several functional assays such as cell viability, apoptotic and motility assays are recommended to be performed on both of the J3T spheres with wild-type gene expression and with increased or decreased expression of a gene of interest. To alter the expression levels of genes of interest in the spheres, one approach is to transfect these spheres with siRNA and short hairpin RNA (shRNA) (to down-regulate gene expression) or with a vector which carries and up-regulates a gene of interest (to up-regulate gene expression) (Doi *et al.* 2003; Li *et al.* 2012).

Although the data obtained from the cDNA microarray analysis indicate that several canonical pathways have a tendency to be up-regulated (e.g. Raf/Mek/Erk and Jak/Stat pathways) or down-regulated (e.g. class I PI3K/Akt/mTOR and IGF-1R pathways) in the J3T spheres, it should be borne in mind that the cDNA microarray is

used to quantify the transcriptional levels of genes of interest, instead of quantifying the active status (phosphoarylation levels) of components of these pathways. Further investigation of the activities, such as phosphorylation status, of these pathways by Western Blot and Reverse-Phase Protein Array can corroborate the current findings.

Reference list

(2010). "Ridaforolimus." Drugs R D **10**(3): 165-78.

Ada-Nguema, A. S., H. Xenias, et al. (2006). "The small GTPase R-Ras regulates organization of actin and drives membrane protrusions through the activity of PLCepsilon." J Cell Sci **119**(Pt 7): 1307-19.

Adachi, M. and K. Imai (2002). "The proapoptotic BH3-only protein BAD transduces cell death signals independently of its interaction with Bcl-2." Cell Death Differ **9**(11): 1240-7.

Aho, T. L., J. Sandholm, et al. (2004). "Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site." FEBS Lett **571**(1-3): 43-9.

Akhtar, L. N. and E. N. Benveniste (2011). "Viral exploitation of host SOCS protein functions." J Virol **85**(5): 1912-21.

Akhtar, N., M. L. Padilla, et al. (2004). "Interleukin-12 inhibits tumor growth in a novel angiogenesis canine hemangiosarcoma xenograft model." Neoplasia **6**(2): 106-16.

Akin, C. (2006). "Molecular diagnosis of mast cell disorders: a paper from the 2005 William Beaumont Hospital Symposium on Molecular Pathology." J Mol Diagn **8**(4): 412-9.

Aksamitiene, E., B. N. Kholodenko, et al. (2010). "PI3K/Akt-sensitive MEK-independent compensatory circuit of ERK activation in ER-positive PI3K-mutant T47D breast cancer cells." Cell Signal **22**(9): 1369-78.

Al-Hajj, M., M. S. Wicha, et al. (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-8.

Al Moustafa, A. E., M. A. Alaoui-Jamali, et al. (2002). "Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells." Oncogene **21**(17): 2634-40.

Alarcon-Vargas, D. and Z. Ronai (2002). "p53-Mdm2--the affair that never ends." Carcinogenesis **23**(4): 541-7.

Alessi, D. R., M. Andjelkovic, et al. (1996). "Mechanism of activation of protein kinase B by insulin and IGF-1." Embo J **15**(23): 6541-51.

Alessi, D. R., F. B. Caudwell, et al. (1996). "Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase." FEBS Lett **399**(3): 333-8.

Alessi, D. R., M. Deak, et al. (1997). "3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase." Curr Biol **7**(10): 776-89.

Alexandre, J., E. Raymond, et al. (1999). "[Rapamycin and CCI-779]." Bull Cancer **86**(10): 808-11.

Almoguera, C., D. Shibata, et al. (1988). "Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes." Cell **53**(4): 549-54.

Altomare, D. A. and J. R. Testa (2005). "Perturbations of the AKT signaling pathway in human cancer." Oncogene **24**(50): 7455-64.

Amantini, C., M. Mosca, et al. (2007). "Capsaicin-induced apoptosis of glioma cells is mediated by TRPV1 vanilloid receptor and requires p38 MAPK activation." J Neurochem **102**(3): 977-90.

Anderson, K., C. Lutz, et al. (2011). "Genetic variegation of clonal architecture and propagating cells in leukaemia." Nature **469**(7330): 356-61.

Andreu-Perez, P., J. Hernandez-Losa, et al. (2010). "Methylthioadenosine (MTA) inhibits melanoma cell proliferation and in vivo tumor growth." BMC Cancer **10**: 265.

Anjum, R. and J. Blenis (2008). "The RSK family of kinases: emerging roles in cellular signalling." Nat Rev Mol Cell Biol **9**(10): 747-58.

Anzai, K., E. Sekine-Suzuki, et al. (2011). "Effectiveness of combined treatment using X-rays and a phosphoinositide 3-kinase inhibitor, ZSTK474, on proliferation of HeLa cells in vitro and in vivo." Cancer Sci **102**(6): 1176-80.

Aoki, H., Y. Kondo, et al. (2008). "Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3." Autophagy **4**(4): 467-75.

Arbab, A. S. (2012). "Activation of alternative pathways of angiogenesis and involvement of stem cells following anti-angiogenesis treatment in glioma." Histol Histopathol **27**(5): 549-57.

Argiris, A., E. Cohen, et al. (2006). "A phase II trial of perifosine, an oral alkylphospholipid, in recurrent or metastatic head and neck cancer." Cancer Biol Ther **5**(7): 766-70.

Asakura, A., P. Seale, et al. (2002). "Myogenic specification of side population cells in skeletal muscle." J Cell Biol **159**(1): 123-34.

Aspenstrom, P. (2010). "Formin-binding proteins: modulators of formin-dependent actin polymerization." Biochim Biophys Acta **1803**(2): 174-82.

Assimakopoulou, M. and J. Varakis (2001). "AP-1 and heat shock protein 27 expression in human astrocytomas." J Cancer Res Clin Oncol **127**(12): 727-32.

Auguste, P., D. B. Gursel, et al. (2001). "Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms." Cancer Res **61**(4): 1717-26.

Avruch, J., X. Long, et al. (2009). "Activation of mTORC1 in two steps: Rheb-GTP activation of catalytic function and increased binding of substrates to raptor." Biochem Soc Trans **37**(Pt 1): 223-6.

Baccelli, I. and A. Trumpp (2012). "The evolving concept of cancer and metastasis stem cells." J Cell Biol **198**(3): 281-93.

Baer, K., I. Lisinski, et al. (2005). "Activation of a GST-tagged AKT2/PKBbeta." Biochim Biophys Acta **1725**(3): 340-7.

Bagley, J. A. and L. Belluscio (2010). "Dynamic imaging reveals that brain-derived neurotrophic factor can independently regulate motility and direction of neuroblasts within the rostral migratory stream." Neuroscience **169**(3): 1449-61.

Bailey, S. M. and J. P. Murnane (2006). "Telomeres, chromosome instability and cancer." Nucleic Acids Res **34**(8): 2408-17.

Baker, S. J., A. C. Preisinger, et al. (1990). "p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis." Cancer Res **50**(23): 7717-22.

Baldwin, R. M., G. M. Barrett, et al. (2010). "Coordination of glioblastoma cell motility by PKCiota." Mol Cancer **9**: 233.

Banin, S., L. Moyal, et al. (1998). "Enhanced phosphorylation of p53 by ATM in response to DNA damage." Science **281**(5383): 1674-7.

Bao, S., Q. Wu, et al. (2006). "Glioma stem cells promote radioresistance by preferential activation of the DNA damage response." Nature **444**(7120): 756-60.

Barbacid, M. (1994). "The Trk family of neurotrophin receptors." J Neurobiol **25**(11): 1386-403.

Barber, D. F., A. Bartolome, et al. (2005). "PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus." Nat Med **11**(9): 933-5.

Bartkowska, K., A. Paquin, et al. (2007). "Trk signaling regulates neural precursor cell proliferation and differentiation during cortical development." Development **134**(24): 4369-80.

Baumann, P., S. Mandl-Weber, et al. (2009). "The novel orally bioavailable inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin, NVP-BEZ235, inhibits growth and proliferation in multiple myeloma." Exp Cell Res **315**(3): 485-97.

Bayle, J., S. Letard, et al. (2004). "Suppressor of cytokine signaling 6 associates with KIT and regulates KIT receptor signaling." J Biol Chem **279**(13): 12249-59.

Becher, O. J., K. M. Peterson, et al. (2008). "IGFBP2 is overexpressed by pediatric malignant astrocytomas and induces the repair enzyme DNA-PK." J Child Neurol **23**(10): 1205-13.

Beckner, M. E., W. Fellows-Mayle, et al. (2010). "Identification of ATP citrate lyase as a positive regulator of glycolytic function in glioblastomas." Int J Cancer **126**(10): 2282-95.

Behrens, A., M. Sibilia, et al. (1999). "Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation." Nat Genet **21**(3): 326-9.

Belfiore, A. and R. Malaguarnera (2011). "Insulin receptor and cancer." Endocr Relat Cancer **18**(4): R125-47.

Bellacosa, A., D. de Feo, et al. (1995). "Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas." Int J Cancer **64**(4): 280-5.

Bellacosa, A., C. C. Kumar, et al. (2005). "Activation of AKT kinases in cancer: implications for therapeutic targeting." Adv Cancer Res **94**: 29-86.

Bender, A., D. Opel, et al. (2011). "PI3K inhibitors prime neuroblastoma cells for chemotherapy by shifting the balance towards pro-apoptotic Bcl-2 proteins and enhanced mitochondrial apoptosis." Oncogene **30**(4): 494-503.

Berens, M. E., A. Giese, et al. (1999). "Allogeneic astrocytoma in immune competent dogs." Neoplasia **1**(2): 107-12.

Beretta, L., A. C. Gingras, et al. (1996). "Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation." Embo J **15**(3): 658-64.

Berndt, N., H. Yang, et al. (2010). "The Akt activation inhibitor TCN-P inhibits Akt phosphorylation by binding to the PH domain of Akt and blocking its recruitment to the plasma membrane." Cell Death Differ **17**(11): 1795-804.

Bertelsen, B. I., S. J. Steine, et al. (2006). "Molecular analysis of the PI3K-AKT pathway in uterine cervical neoplasia: frequent PIK3CA amplification and AKT phosphorylation." Int J Cancer **118**(8): 1877-83.

Bianchini, A., M. Loiarro, et al. (2008). "Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells." Carcinogenesis **29**(12): 2279-88.

Biondi, R. M., A. Kieloch, et al. (2001). "The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB." Embo J **20**(16): 4380-90.

Birle, D., N. Bottini, et al. (2002). "Negative feedback regulation of the tumor suppressor PTEN by phosphoinositide-induced serine phosphorylation." J Immunol **169**(1): 286-91.

Bishton, M., M. Kenealy, et al. (2007). "Epigenetic targets in hematological malignancies: combination therapies with HDACis and demethylating agents." Expert Rev Anticancer Ther **7**(10): 1439-49.

Bjornsti, M. A. and P. J. Houghton (2004). "The TOR pathway: a target for cancer therapy." Nat Rev Cancer **4**(5): 335-48.

Blaese, R. M., K. W. Culver, et al. (1995). "T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years." Science **270**(5235): 475-80.

Blair, A., D. E. Hogge, et al. (1997). "Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo." Blood **89**(9): 3104-12.

Blair, A., D. E. Hogge, et al. (1998). "Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR." Blood **92**(11): 4325-35.

Blair, A. and H. J. Sutherland (2000). "Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD117)." Exp Hematol **28**(6): 660-71.

Bleau, A. M., D. Hambardzumyan, et al. (2009). "PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells." Cell Stem Cell **4**(3): 226-35.

Blommaart, E. F., J. J. Luiken, et al. (1995). "Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes." J Biol Chem **270**(5): 2320-6.

Boivin, D., D. Labbe, et al. (2009). "The stem cell marker CD133 (prominin-1) is phosphorylated on cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases." Biochemistry **48**(18): 3998-4007.

Bonnet, D. and J. E. Dick (1997). "Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell." Nat Med **3**(7): 730-7.

Bonni, A., Y. Sun, et al. (1997). "Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway." Science **278**(5337): 477-83.

Borovski, T., E. M. F. De Sousa, et al. (2011). "Cancer stem cell niche: the place to be." Cancer Res **71**(3): 634-9.

Bosotti, R., A. Isacchi, et al. (2000). "FAT: a novel domain in PIK-related kinases." Trends Biochem Sci **25**(5): 225-7.

Bottenstein, J. E. and G. H. Sato (1979). "Growth of a rat neuroblastoma cell line in serum-free supplemented medium." Proc Natl Acad Sci U S A **76**(1): 514-7.

Boulalas, I., A. Zaravinos, et al. (2009). "Activation of RAS family genes in urothelial carcinoma." J Urol **181**(5): 2312-9.

Boulay, A., S. Zumstein-Mecker, et al. (2004). "Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells." Cancer Res **64**(1): 252-61.

Boulbes, D., C. H. Chen, et al. (2010). "Rictor phosphorylation on the Thr-1135 site does not require mammalian target of rapamycin complex 2." Mol Cancer Res **8**(6): 896-906.

Braess, J., C. Wegendt, et al. (1999). "Leukaemic blasts differ from normal bone marrow mononuclear cells and CD34+ haemopoietic stem cells in their metabolism of cytosine arabinoside." Br J Haematol **105**(2): 388-93.

Braunstein, S., K. Karpisheva, et al. (2007). "A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer." Mol Cell **28**(3): 501-12.

Bray F, Ren JS, et al. (2010). "Estimates of global cancer prevalence in 2008 for 27 sites in the adult population, submitted. ."

Brodbeck, D., P. Cron, et al. (1999). "A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain." J Biol Chem **274**(14): 9133-6.

Brognard, J., A. S. Clark, et al. (2001). "Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation." Cancer Res **61**(10): 3986-97.

Brunet, A., A. Bonni, et al. (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." Cell **96**(6): 857-68.

Bryder, D., D. J. Rossi, et al. (2006). "Hematopoietic stem cells: the paradigmatic tissue-specific stem cell." Am J Pathol **169**(2): 338-46.

Buck, E., A. Eyzaguirre, et al. (2006). "Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors." Mol Cancer Ther **5**(11): 2676-84.

Buie, L. W. and J. Valgus (2008). "Bevacizumab: a treatment option for recurrent glioblastoma multiforme." Ann Pharmacother **42**(10): 1486-90.

Bunting, K. D. (2002). "ABC transporters as phenotypic markers and functional regulators of stem cells." Stem Cells **20**(1): 11-20.

Burdon, T., A. Smith, et al. (2002). "Signalling, cell cycle and pluripotency in embryonic stem cells." Trends Cell Biol **12**(9): 432-8.

Burgess, D. J., J. Doles, et al. (2008). "Topoisomerase levels determine chemotherapy response in vitro and in vivo." Proc Natl Acad Sci U S A **105**(26): 9053-8.

Burnett, P. E., R. K. Barrow, et al. (1998). "RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1." Proc Natl Acad Sci U S A **95**(4): 1432-7.

Byfield, M. P., J. T. Murray, et al. (2005). "hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase." J Biol Chem **280**(38): 33076-82.

Byun, D. S., K. Cho, et al. (2003). "Frequent monoallelic deletion of PTEN and its reciprocal associatioin with PIK3CA amplification in gastric carcinoma." Int J Cancer **104**(3): 318-27.

Cachero, T. G., A. D. Morielli, et al. (1998). "The small GTP-binding protein RhoA regulates a delayed rectifier potassium channel." Cell **93**(6): 1077-85.

Callaghan, T., M. Antczak, et al. (1993). "A complete description of the EGF-receptor exon structure: implication in oncogenic activation and domain evolution." Oncogene **8**(11): 2939-48.

Calleja, V., D. Alcor, et al. (2007). "Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo." PLoS Biol **5**(4): e95.

Calleja, V., M. Laguerre, et al. (2009). "Role of a novel PH-kinase domain interface in PKB/Akt regulation: structural mechanism for allosteric inhibition." PLoS Biol **7**(1): e17.

Calzolari, F. and P. Malatesta (2010). "Recent insights into PDGF-induced gliomagenesis." Brain Pathol **20**(3): 527-38.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-7.

Cao, L., W. Cao, et al. (2008). "Identification of 14-3-3 protein isoforms in human astrocytoma by immunohistochemistry." Neurosci Lett **432**(2): 94-9.

Cao, W., X. Yang, et al. (2010). "Targeting 14-3-3 protein, difopein induces apoptosis of human glioma cells and suppresses tumor growth in mice." Apoptosis **15**(2): 230-41.

Cardone, M. H., N. Roy, et al. (1998). "Regulation of cell death protease caspase-9 by phosphorylation." Science **282**(5392): 1318-21.

Carpten, J. D., A. L. Faber, et al. (2007). "A transforming mutation in the pleckstrin homology domain of AKT1 in cancer." Nature **448**(7152): 439-44.

Carracedo, A., L. Ma, et al. (2008). "Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer." J Clin Invest **118**(9): 3065-74.

Carroll, R. S., J. Zhang, et al. (1999). "KDR activation in astrocytic neoplasms." Cancer **86**(7): 1335-41.

Cartier, N. and P. Aubourg (2010). "Hematopoietic stem cell transplantation and hematopoietic stem cell gene therapy in X-linked adrenoleukodystrophy." Brain Pathol **20**(4): 857-62.

Casamayor, A., N. A. Morrice, et al. (1999). "Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo." Biochem J **342 (Pt 2)**: 287-92.

Castellano, E. and J. Downward (2011). "RAS Interaction with PI3K: More Than Just Another Effector Pathway." Genes Cancer **2**(3): 261-74.

Castro, J. R., J. A. Costoya, et al. (2000). "Expression of growth hormone receptor in the human brain." Neurosci Lett **281**(2-3): 147-50.

Chabner, B. A. and D. L. Longo (2001). Cancer Chemotherapy and Biotherapy: Principles and Practice, Lippincott Williams & Wilkins, Philadelphia.

Charest, A., E. W. Wilker, et al. (2006). "ROS fusion tyrosine kinase activates a SH2 domain-containing phosphatase-2/phosphatidylinositol 3-kinase/mammalian target of rapamycin signaling axis to form glioblastoma in mice." Cancer Res **66**(15): 7473-81.

Charles, N., T. Ozawa, et al. (2010). "Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells." Cell Stem Cell **6**(2): 141-52.

Chaturvedi, D., X. Gao, et al. (2009). "Rapamycin induces transactivation of the EGFR and increases cell survival." Oncogene **28**(9): 1187-96.

Chen, C. H., T. Shaikenov, et al. (2011). "ER stress inhibits mTORC2 and Akt signaling through GSK-3beta-mediated phosphorylation of rictor." Sci Signal **4**(161): ra10.

Chen, C. L., Y. T. Hsieh, et al. (2007). "Phosphorylation of adducin by protein kinase Cdelta promotes cell motility." J Cell Sci **120**(Pt 7): 1157-67.

Chen, J. H., S. M. Huang, et al. (2011). "Ghrelin induces cell migration through GHS-R, CaMKII, AMPK, and NF-kappaB signaling pathway in glioma cells." J Cell Biochem **112**(10): 2931-41.

Chen, T. C., D. R. Hinton, et al. (1998). "Up-regulation of the cAMP/PKA pathway inhibits proliferation, induces differentiation, and leads to apoptosis in malignant gliomas." Lab Invest **78**(2): 165-74.

Chen, W. S., P. Z. Xu, et al. (2001). "Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene." Genes Dev **15**(17): 2203-8.

Chen, W. W., D. C. Chan, et al. (2005). "Pim family kinases enhance tumor growth of prostate cancer cells." Mol Cancer Res **3**(8): 443-51.

Chen, X. G., F. Liu, et al. (2010). "Rapamycin regulates Akt and ERK phosphorylation through mTORC1 and mTORC2 signaling pathways." Mol Carcinog **49**(6): 603-10.

Chen, Y. and X. Y. Du (2007). "Functional properties and intracellular signaling of CCN1/Cyr61." J Cell Biochem **100**(6): 1337-45.

Chen, Y., B. Stevens, et al. (2008). "NS21: re-defined and modified supplement B27 for neuronal cultures." J Neurosci Methods **171**(2): 239-47.

Cheng, J. Q., C. W. Lindsley, et al. (2005). "The Akt/PKB pathway: molecular target for cancer drug discovery." Oncogene **24**(50): 7482-92.

Cheng, J. Q., B. Ruggeri, et al. (1996). "Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA." Proc Natl Acad Sci U S A **93**(8): 3636-41.

Chernausek, S. D., M. A. Murray, et al. (1993). "Expression of insulin-like growth factor binding protein-4 (IGFBP-4) by rat neural cells--comparison to other IGFBPs." Regul Pept **48**(1-2): 123-32.

Cheung, T. H., K. W. Lo, et al. (2004). "Epigenetic and genetic alternation of PTEN in cervical neoplasm." Gynecol Oncol **93**(3): 621-7.

Chiang, G. G. and R. T. Abraham (2007). "Targeting the mTOR signaling network in cancer." Trends Mol Med **13**(10): 433-42.

Chiang, S. H., J. C. Hou, et al. (2002). "Cloning and functional characterization of related TC10 isoforms, a subfamily of Rho proteins involved in insulin-stimulated glucose transport." J Biol Chem **277**(15): 13067-73.

Chiariello, E., L. Roz, et al. (1998). "PTEN/MMAC1 mutations in primary glioblastomas and short-term cultures of malignant gliomas." Oncogene **16**(4): 541-5.

Chiarini, F., C. Grimaldi, et al. (2010). "Activity of the novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 against T-cell acute lymphoblastic leukemia." Cancer Res **70**(20): 8097-107.

Chiba, T., K. Kita, et al. (2006). "Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties." Hepatology **44**(1): 240-51.

Cho, H., J. Mu, et al. (2001). "Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta)." Science **292**(5522): 1728-31.

Cho, H., J. L. Thorvaldsen, et al. (2001). "Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice." J Biol Chem **276**(42): 38349-52.

Choe, G., S. Horvath, et al. (2003). "Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo." Cancer Res **63**(11): 2742-6.

Choo, E. F., B. Leake, et al. (2000). "Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes." Drug Metab Dispos **28**(6): 655-60.

Chorobik, P. and J. Marcinkiewicz (2011). "Therapeutic vaccines based on genetically modified Salmonella: a novel strategy in cancer immunotherapy." Pol Arch Med Wewn **121**(12): 461-6.

Choy, L. and R. Derynck (1998). "The type II transforming growth factor (TGF)-beta receptor-interacting protein TRIP-1 acts as a modulator of the TGF-beta response." J Biol Chem **273**(47): 31455-62.

Chresta, C. M., B. R. Davies, et al. (2010). "AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity." Cancer Res **70**(1): 288-98.

Christofori, G. and H. Semb (1999). "The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene." Trends Biochem Sci **24**(2): 73-6.

Chua, C., N. Zaiden, et al. (2008). "Characterization of a side population of astrocytoma cells in response to temozolomide." J Neurosurg **109**(5): 856-66.

Chuaqui, R. F., R. F. Bonner, et al. (2002). "Post-analysis follow-up and validation of microarray experiments." Nat Genet **32 Suppl**: 509-14.

Ciesielski, M. J. and R. A. Fenstermaker (2000). "Oncogenic epidermal growth factor receptor mutants with tandem duplication: gene structure and effects on receptor function." Oncogene **19**(6): 810-20.

Ciuffreda, L., C. Di Sanza, et al. (2010). "The mTOR pathway: a new target in cancer therapy." Curr Cancer Drug Targets **10**(5): 484-95.

Clark, A. S., K. West, et al. (2002). "Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells." Mol Cancer Ther **1**(9): 707-17.

Classon, M. and E. Harlow (2002). "The retinoblastoma tumour suppressor in development and cancer." Nat Rev Cancer **2**(12): 910-7.

Clayton, H., I. Titley, et al. (2004). "Growth and differentiation of progenitor/stem cells derived from the human mammary gland." Exp Cell Res **297**(2): 444-60.

Cobb, W. R., A. E. Bogden, et al. (1983). "Activity of two phase I drugs, homoharringtonine and tricyclic nucleotide, against surgical explants of human tumors in the 6-day subrenal capsule assay." Cancer Treat Rep **67**(2): 173-8.

Coe, I. R., L. Yao, et al. (1996). "The role of protein kinase C in cellular tolerance to ethanol." J Biol Chem **271**(46): 29468-72.

Cohen, P. T. (2002). "Protein phosphatase 1--targeted in many directions." J Cell Sci **115**(Pt 2): 241-56.

Collins, A. T., P. A. Berry, et al. (2005). "Prospective identification of tumorigenic prostate cancer stem cells." Cancer Res **65**(23): 10946-51.

Cooper, G. M. (2000). The cell: a molecular approach. Sunderland, Massachusetts, USA, Sinauer Associates, Inc.

Cordon-Cardo, C., J. P. O'Brien, et al. (1989). "Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites." Proc Natl Acad Sci U S A **86**(2): 695-8.

Cotsarelis, G., S. Z. Cheng, et al. (1989). "Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells." Cell **57**(2): 201-9.

Counter, C. M., A. A. Avilion, et al. (1992). "Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity." Embo J **11**(5): 1921-9.

Cox, D., M. Brennan, et al. (2010). "Integrins as therapeutic targets: lessons and opportunities." Nat Rev Drug Discov **9**(10): 804-20.

Cross, D. A., D. R. Alessi, et al. (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." Nature **378**(6559): 785-9.

Cully, M., H. You, et al. (2006). "Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis." Nat Rev Cancer **6**(3): 184-92.

Dal Col, J., P. Zancai, et al. (2008). "Distinct functional significance of Akt and mTOR constitutive activation in mantle cell lymphoma." Blood **111**(10): 5142-51.

Damen, J. E., L. Liu, et al. (1996). "The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase." Proc Natl Acad Sci U S A **93**(4): 1689-93.

Das, G., A. Shiras, et al. (2011). "Rictor regulates MMP-9 activity and invasion through Raf-1-MEK-ERK signaling pathway in glioma cells." Mol Carcinog **50**(6): 412-23.

Datta, S. R., H. Dudek, et al. (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery." Cell **91**(2): 231-41.

Davies, M. A., K. Stemke-Hale, et al. (2008). "A novel AKT3 mutation in melanoma tumours and cell lines." Br J Cancer **99**(8): 1265-8.

Davies, S. P., H. Reddy, et al. (2000). "Specificity and mechanism of action of some commonly used protein kinase inhibitors." Biochem J **351**(Pt 1): 95-105.

De Benedetti, A. and J. R. Graff (2004). "eIF-4E expression and its role in malignancies and metastases." Oncogene **23**(18): 3189-99.

De Benedetti, A., S. Joshi-Barve, et al. (1991). "Expression of antisense RNA against initiation factor eIF-4E mRNA in HeLa cells results in lengthened cell division times, diminished translation rates, and reduced levels of both eIF-4E and the p220 component of eIF-4F." Mol Cell Biol **11**(11): 5435-45.

De Nadai, C., P. Huitorel, et al. (1998). "Effect of wortmannin, an inhibitor of phosphatidylinositol 3-kinase, on the first mitotic divisions of the fertilized sea urchin egg." J Cell Sci **111 (Pt 17)**: 2507-18.

de Toledo, M., F. Senic-Matuglia, et al. (2003). "The GTP/GDP cycling of rho GTPase TCL is an essential regulator of the early endocytic pathway." Mol Biol Cell **14**(12): 4846-56.

DeCaprio, J. A., J. W. Ludlow, et al. (1988). "SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene." Cell **54**(2): 275-83.

Demuth, T., L. B. Reavie, et al. (2007). "MAP-ing glioma invasion: mitogen-activated protein kinase kinase 3 and p38 drive glioma invasion and progression and predict patient survival." Mol Cancer Ther **6**(4): 1212-22.

Deng, T. and M. Karin (1994). "c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK." Nature **371**(6493): 171-5.

Desai, B. N., B. R. Myers, et al. (2002). "FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction." Proc Natl Acad Sci U S A **99**(7): 4319-24.

Desai, S., P. Pillai, et al. (2011). "PKC-iota promotes glioblastoma cell survival by phosphorylating and inhibiting BAD through a phosphatidylinositol 3-kinase pathway." Biochim Biophys Acta **1813**(6): 1190-7.

Desai, S. R., P. P. Pillai, et al. (2012). "Regulation of Cdk7 activity through a phosphatidylinositol (3)-kinase/PKC-iota-mediated signaling cascade in glioblastoma." Carcinogenesis **33**(1): 10-9.

DeVinney, R. and W. M. Gold (1990). "Establishment of two dog mastocytoma cell lines in continuous culture." Am J Respir Cell Mol Biol **3**(5): 413-20.

di Tomaso, E., N. London, et al. (2009). "PDGF-C induces maturation of blood vessels in a model of glioblastoma and attenuates the response to anti-VEGF treatment." PLoS One **4**(4): e5123.

Diehn, M., R. W. Cho, et al. (2009). "Association of reactive oxygen species levels and radioresistance in cancer stem cells." Nature **458**(7239): 780-3.

Dietrich, J., A. D. Norden, et al. (2008). "Emerging antiangiogenic treatments for gliomas - efficacy and safety issues." Curr Opin Neurol **21**(6): 736-44.

Dimmeler, S., I. Fleming, et al. (1999). "Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation." Nature **399**(6736): 601-5.

Ding, L., G. Getz, et al. (2008). "Somatic mutations affect key pathways in lung adenocarcinoma." Nature **455**(7216): 1069-75.

Dinudom, A., K. F. Harvey, et al. (1998). "Nedd4 mediates control of an epithelial Na+ channel in salivary duct cells by cytosolic Na+." Proc Natl Acad Sci U S A **95**(12): 7169-73.

Dittmer, D., S. Pati, et al. (1993). "Gain of function mutations in p53." Nat Genet **4**(1): 42-6.

Dixit, D., V. Sharma, et al. (2012). "Inhibition of Casein kinase-2 induces p53-dependent cell cycle arrest and sensitizes glioblastoma cells to tumor necrosis factor (TNFalpha)-induced apoptosis through SIRT1 inhibition." Cell Death Dis **3**: e271.

Doi, N., S. Zenno, et al. (2003). "Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors." Curr Biol **13**(1): 41-6.

Domin, J., F. Pages, et al. (1997). "Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin." Biochem J **326 (Pt 1)**: 139-47.

Domina, A. M., J. A. Vrana, et al. (2004). "MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol." Oncogene **23**(31): 5301-15.

Dorman, D. C., K. A. Coddington, et al. (1990). "5-Fluorouracil toxicosis in the dog." J Vet Intern Med **4**(5): 254-7.

Dorrello, N. V., A. Peschiaroli, et al. (2006). "S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth." Science **314**(5798): 467-71.

Dorsam, R. T. and J. S. Gutkind (2007). "G-protein-coupled receptors and cancer." Nat Rev Cancer **7**(2): 79-94.

Doukas, J., W. Wrasidlo, et al. (2006). "Phosphoinositide 3-kinase gamma/delta inhibition limits infarct size after myocardial ischemia/reperfusion injury." Proc Natl Acad Sci U S A **103**(52): 19866-71.

Drexler, H. G. (1996). "Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells." Leukemia **10**(4): 588-99.

Du, K. and P. N. Tsichlis (2005). "Regulation of the Akt kinase by interacting proteins." Oncogene **24**(50): 7401-9.

Dubois, L., M. G. Magagnin, et al. (2009). "Inhibition of 4E-BP1 sensitizes U87 glioblastoma xenograft tumors to irradiation by decreasing hypoxia tolerance." Int J Radiat Oncol Biol Phys **73**(4): 1219-27.

Dubrovska, A., S. Kim, et al. (2009). "The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations." Proc Natl Acad Sci U S A **106**(1): 268-73.

Dunlap, S. M., J. Celestino, et al. (2007). "Insulin-like growth factor binding protein 2 promotes glioma development and progression." Proc Natl Acad Sci U S A **104**(28): 11736-41.

Earnshaw, W. C., L. M. Martins, et al. (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochem **68**: 383-424.

Easton, R. M., H. Cho, et al. (2005). "Role for Akt3/protein kinase Bgamma in attainment of normal brain size." Mol Cell Biol **25**(5): 1869-78.

Egeblad, M. and Z. Werb (2002). "New functions for the matrix metalloproteinases in cancer progression." Nat Rev Cancer **2**(3): 161-74.

Eguez, L., A. Lee, et al. (2005). "Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein." Cell Metab **2**(4): 263-72.

El-Salem, M., P. N. Raghunath, et al. (2007). "Constitutive activation of mTOR signaling pathway in post-transplant lymphoproliferative disorders." Lab Invest **87**(1): 29-39.

Elmore, S. (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.

Else, R. W., M. Norval, et al. (1982). "The characteristics of a canine mammary carcinoma cell line, REM 134." Br J Cancer **46**(4): 675-81.

Engelman, J. A. (2009). "Targeting PI3K signalling in cancer: opportunities, challenges and limitations." Nat Rev Cancer **9**(8): 550-62.

Engelman, J. A., L. Chen, et al. (2008). "Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers." Nat Med **14**(12): 1351-6.

Engelman, J. A., J. Luo, et al. (2006). "The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism." Nat Rev Genet **7**(8): 606-19.

Eyler, C. E., W. C. Foo, et al. (2008). "Brain cancer stem cells display preferential sensitivity to Akt inhibition." Stem Cells **26**(12): 3027-36.

Eyler, C. E., Q. Wu, et al. (2011). "Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2." Cell **146**(1): 53-66.

Faivre, S., G. Kroemer, et al. (2006). "Current development of mTOR inhibitors as anticancer agents." Nat Rev Drug Discov **5**(8): 671-88.

Fan, Q. W., C. Cheng, et al. (2010). "Akt and autophagy cooperate to promote survival of drug-resistant glioma." Sci Signal **3**(147): ra81.

Fan, X., L. Khaki, et al. (2010). "NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts." Stem Cells **28**(1): 5-16.

Fan, X., W. Matsui, et al. (2006). "Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors." Cancer Res **66**(15): 7445-52.

Fang, D., T. K. Nguyen, et al. (2005). "A tumorigenic subpopulation with stem cell properties in melanomas." Cancer Res **65**(20): 9328-37.

Fanton, C. P., M. McMahon, et al. (2001). "Dual growth arrest pathways in astrocytes and astrocytic tumors in response to Raf-1 activation." J Biol Chem **276**(22): 18871-7.

Fata, F., E. O'Reilly, et al. (1999). "Paclitaxel in the treatment of patients with angiosarcoma of the scalp or face." Cancer **86**(10): 2034-7.

Feldman, M. E., B. Apsel, et al. (2009). "Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2." PLoS Biol **7**(2): e38.

Feng, H., B. Hu, et al. (2011). "Activation of Rac1 by Src-dependent phosphorylation of Dock180(Y1811) mediates PDGFRalpha-stimulated glioma tumorigenesis in mice and humans." J Clin Invest **121**(12): 4670-84.

Feng, Z., H. Zhang, et al. (2005). "The coordinate regulation of the p53 and mTOR pathways in cells." Proc Natl Acad Sci U S A **102**(23): 8204-9.

Fenouille, N., A. Puissant, et al. (2011). "SPARC functions as an anti-stress factor by inactivating p53 through Akt-mediated MDM2 phosphorylation to promote melanoma cell survival." Oncogene.

Fenton, T. R. and I. T. Gout (2011). "Functions and regulation of the 70kDa ribosomal S6 kinases." Int J Biochem Cell Biol **43**(1): 47-59.

Ferlay J, Shin HR, et al. (2010). "GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]." Lyon, France: International Agency for Research on Cancer. Lyon, France: International Agency for Research on Cancer.

Fernandez-Medarde, A. and E. Santos (2011). "Ras in cancer and developmental diseases." Genes Cancer **2**(3): 344-58.

Ferrari, S., H. R. Bandi, et al. (1991). "Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites." J Biol Chem **266**(33): 22770-5.

Ferrua, F., I. Brigida, et al. (2010). "Update on gene therapy for adenosine deaminase-deficient severe combined immunodeficiency." Curr Opin Allergy Clin Immunol **10**(6): 551-6.

Fidler, I. J. and M. L. Kripke (1977). "Metastasis results from preexisting variant cells within a malignant tumor." Science **197**(4306): 893-5.

Fields, A. P. and R. P. Regala (2007). "Protein kinase C iota: human oncogene, prognostic marker and therapeutic target." Pharmacol Res **55**(6): 487-97.

Filippa, N., C. L. Sable, et al. (1999). "Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase." Mol Cell Biol **19**(7): 4989-5000.

Findlay, G. M., L. Yan, et al. (2007). "A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling." Biochem J **403**(1): 13-20.

Finora, K. (2003). "Common paraneoplastic syndromes." Clin Tech Small Anim Pract **18**(2): 123-6.

Fioretos, T., I. Panagopoulos, et al. (2001). "Fusion of the BCR and the fibroblast growth factor receptor-1 (FGFR1) genes as a result of t(8;22)(p11;q11) in a myeloproliferative disorder: the first fusion gene involving BCR but not ABL." Genes Chromosomes Cancer **32**(4): 302-10.

Folkins, C., S. Man, et al. (2007). "Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors." Cancer Res **67**(8): 3560-4.

Folkman, J. (1971). "Tumor angiogenesis: therapeutic implications." N Engl J Med **285**(21): 1182-6.

Forbes, S. A., N. Bindal, et al. (2011). "COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer." Nucleic Acids Res **39**(Database issue): D945-50.

Fox, C. J., P. S. Hammerman, et al. (2003). "The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor." Genes Dev **17**(15): 1841-54.

Fransson, A., A. Ruusala, et al. (2003). "Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis." J Biol Chem **278**(8): 6495-502.

Fransson, S., A. Ruusala, et al. (2006). "The atypical Rho GTPases Miro-1 and Miro-2 have essential roles in mitochondrial trafficking." Biochem Biophys Res Commun **344**(2): 500-10.

Fraser, C. S., K. E. Berry, et al. (2007). "eIF3j is located in the decoding center of the human 40S ribosomal subunit." Mol Cell **26**(6): 811-9.

Fraser, C. S., J. Y. Lee, et al. (2004). "The j-subunit of human translation initiation factor eIF3 is required for the stable binding of eIF3 and its subcomplexes to 40 S ribosomal subunits in vitro." J Biol Chem **279**(10): 8946-56.

Frias, M. A., C. C. Thoreen, et al. (2006). "mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s." Curr Biol **16**(18): 1865-70.

Fromm, M. F. (2000). "P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs." Int J Clin Pharmacol Ther **38**(2): 69-74.

Fu, Y. and C. S. Rubin (2011). "Protein kinase D: coupling extracellular stimuli to the regulation of cell physiology." EMBO Rep **12**(8): 785-96.

Fukushima, T., T. Tezuka, et al. (2007). "Silencing of insulin-like growth factor-binding protein-2 in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24." J Biol Chem **282**(25): 18634-44.

Fumagalli, S. and G. Thomas (2000). "S6 phosphorylation and signal transduction." In: Translational Control of Gene Expression (Sonenberg, N., Hershey, J.W.B. & Mathews, M. B., eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Furitsu, T., T. Tsujimura, et al. (1993). "Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product." J Clin Invest **92**(4): 1736-44.

Furnari, F. B., T. Fenton, et al. (2007). "Malignant astrocytic glioma: genetics, biology, and paths to treatment." Genes Dev **21**(21): 2683-710.

Furstenberger, G. and H. J. Senn (2002). "Insulin-like growth factors and cancer." Lancet Oncol **3**(5): 298-302.

Furukawa, T., S. Mukherjee, et al. (2000). "rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells." Neuron **26**(2): 383-94.

Gama, A., F. Gartner, et al. (2009). "Immunohistochemical expression of Epidermal Growth Factor Receptor (EGFR) in canine mammary tissues." Res Vet Sci **87**(3): 432-7.

Gan, H. T., M. Tham, et al. (2011). "Identification of ApoE as an autocrine/paracrine factor that stimulates neural stem cell survival via MAPK/ERK signaling pathway." J Neurochem **117**(3): 565-78.

Garcia-Martinez, J. M. and D. R. Alessi (2008). "mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1)." Biochem J **416**(3): 375-85.

Garcia-Morales, P., E. Carrasco-Garcia, et al. (2007). "Inhibition of Hsp90 function by ansamycins causes downregulation of cdc2 and cdc25c and G(2)/M arrest in glioblastoma cell lines." Oncogene **26**(51): 7185-93.

Gardai, S. J., D. A. Hildeman, et al. (2004). "Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils." J Biol Chem **279**(20): 21085-95.

Geng, L., E. Donnelly, et al. (2001). "Inhibition of vascular endothelial growth factor receptor signaling leads to reversal of tumor resistance to radiotherapy." Cancer Res **61**(6): 2413-9.

Ghobrial, I. M., A. Roccaro, et al. (2010). "Clinical and translational studies of a phase II trial of the novel oral Akt inhibitor perifosine in relapsed or relapsed/refractory Waldenstrom's macroglobulinemia." Clin Cancer Res **16**(3): 1033-41.

Gibbs, C. P., V. G. Kukekov, et al. (2005). "Stem-like cells in bone sarcomas: implications for tumorigenesis." Neoplasia **7**(11): 967-76.

Gilley, J., P. J. Coffer, et al. (2003). "FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons." J Cell Biol **162**(4): 613-22.

Gills, J. J. and P. A. Dennis (2009). "Perifosine: update on a novel Akt inhibitor." Curr Oncol Rep **11**(2): 102-10.

Ginestier, C., M. H. Hur, et al. (2007). "ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome." Cell Stem Cell **1**(5): 555-67.

Gingras, A. C., S. G. Kennedy, et al. (1998). "4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway." Genes Dev **12**(4): 502-13.

Gingras, A. C., B. Raught, et al. (1999). "eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation." Annu Rev Biochem **68**: 913-63.

Gingras, A. C., Y. Svitkin, et al. (1996). "Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus." Proc Natl Acad Sci U S A **93**(11): 5578-83.

Gleixner, K. V., L. Rebuzzi, et al. (2007). "Synergistic antiproliferative effects of KIT tyrosine kinase inhibitors on neoplastic canine mast cells." Exp Hematol **35**(10): 1510-21.

Glick, R. P., R. Gettleman, et al. (1989). "Insulin and insulin-like growth factor I in brain tumors: binding and in vitro effects." Neurosurgery **24**(6): 791-7.

Godlewski, J., M. O. Nowicki, et al. (2010). "MicroRNA-451 regulates LKB1/AMPK signaling and allows adaptation to metabolic stress in glioma cells." Mol Cell **37**(5): 620-32.

Gogineni, V. R., A. K. Nalla, et al. (2011). "alpha3beta1 integrin promotes radiation-induced migration of meningioma cells." Int J Oncol **38**(6): 1615-24.

Gonelli, A., C. Mischiati, et al. (2009). "Perspectives of protein kinase C (PKC) inhibitors as anti-cancer agents." Mini Rev Med Chem **9**(4): 498-509.

Goodell, M. A., K. Brose, et al. (1996). "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo." J Exp Med **183**(4): 1797-806.

Goodwin, C. R., B. Lal, et al. (2010). "Cyr61 mediates hepatocyte growth factor-dependent tumor cell growth, migration, and Akt activation." Cancer Res **70**(7): 2932-41.

Gordon, I. K., F. Ye, et al. (2008). "Evaluation of the mammalian target of rapamycin pathway and the effect of rapamycin on target expression and cellular proliferation in osteosarcoma cells from dogs." Am J Vet Res **69**(8): 1079-84.

Gorgoulis, V. G., L. V. Vassiliou, et al. (2005). "Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions." Nature **434**(7035): 907-13.

Gould, E. (2007). "How widespread is adult neurogenesis in mammals?" Nat Rev Neurosci **8**(6): 481-8.

Goulet, C. C., K. A. Volk, et al. (1998). "Inhibition of the epithelial Na+ channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome." J Biol Chem **273**(45): 30012-7.

Graziano, S. L., G. P. Gamble, et al. (1999). "Prognostic significance of K-ras codon 12 mutations in patients with resected stage I and II non-small-cell lung cancer." J Clin Oncol **17**(2): 668-75.

Greenblatt, M. S., W. P. Bennett, et al. (1994). "Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis." Cancer Res **54**(18): 4855-78.

Grimshaw, M. J., L. Cooper, et al. (2008). "Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells." Breast Cancer Res **10**(3): R52.

Griner, E. M. and M. G. Kazanietz (2007). "Protein kinase C and other diacylglycerol effectors in cancer." Nat Rev Cancer **7**(4): 281-94.

Groszer, M., R. Erickson, et al. (2001). "Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo." Science **294**(5549): 2186-9.

Grunberger, G., W. L. Lowe, Jr., et al. (1986). "Insulin receptor of human cerebral gliomas. Structure and function." J Clin Invest **77**(3): 997-1005.

Grzmil, M., P. Morin, Jr., et al. (2011). "MAP kinase-interacting kinase 1 regulates SMAD2-dependent TGF-beta signaling pathway in human glioblastoma." Cancer Res **71**(6): 2392-402.

Gschwendt, M. (1999). "Protein kinase C delta." Eur J Biochem **259**(3): 555-64.

Gu, F., R. Hata, et al. (2005). "Suppression of Stat3 promotes neurogenesis in cultured neural stem cells." J Neurosci Res **81**(2): 163-71.

Gu, X., L. Jones, et al. (2005). "Expression of eukaryotic initiation factor 4E in astrocytic tumors." Appl Immunohistochem Mol Morphol **13**(2): 178-83.

Guan, J. L. (2010). "Integrin signaling through FAK in the regulation of mammary stem cells and breast cancer." IUBMB Life **62**(4): 268-76.

Guan, K. L., C. Figueroa, et al. (2000). "Negative regulation of the serine/threonine kinase B-Raf by Akt." J Biol Chem **275**(35): 27354-9.

Guasch, G., G. J. Mack, et al. (2000). "FGFR1 is fused to the centrosome-associated protein CEP110 in the 8p12 stem cell myeloproliferative disorder with t(8;9)(p12;q33)." Blood **95**(5): 1788-96.

Guasch, G., C. Popovici, et al. (2003). "Endogenous retroviral sequence is fused to FGFR1 kinase in the 8p12 stem-cell myeloproliferative disorder with t(8;19)(p12;q13.3)." Blood **101**(1): 286-8.

Gulati, P., L. D. Gaspers, et al. (2008). "Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34." Cell Metab **7**(5): 456-65.

Gunther, H. S., N. O. Schmidt, et al. (2008). "Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria." Oncogene **27**(20): 2897-909.

Guo, D., I. J. Hildebrandt, et al. (2009). "The AMPK agonist AICAR inhibits the growth of EGFRvIII-expressing glioblastomas by inhibiting lipogenesis." Proc Natl Acad Sci U S A **106**(31): 12932-7.

Gupta, S., A. R. Ramjaun, et al. (2007). "Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice." Cell **129**(5): 957-68.

Gwinn, D. M., D. B. Shackelford, et al. (2008). "AMPK phosphorylation of raptor mediates a metabolic checkpoint." Mol Cell **30**(2): 214-26.

Hafeez, B. B., W. Zhong, et al. (2011). "Genetic ablation of PKC epsilon inhibits prostate cancer development and metastasis in transgenic mouse model of prostate adenocarcinoma." Cancer Res **71**(6): 2318-27.

Hagemann, C., J. Gloger, et al. (2009). "RAF expression in human astrocytic tumors." Int J Mol Med **23**(1): 17-31.

Hagerstrand, D., M. B. Lindh, et al. (2010). "PI3K/PTEN/Akt pathway status affects the sensitivity of high-grade glioma cell cultures to the insulin-like growth factor-1 receptor inhibitor NVP-AEW541." Neuro Oncol **12**(9): 967-75.

Han, C., Z. Gong, et al. (2011). "Mechanism of monoclonal antibody-coupled Staphylococcus superantigen-A induced apoptosis in human bladder cancer cells." Cell Biochem Biophys **61**(3): 679-84.

Han, J., K. Luby-Phelps, et al. (1998). "Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav." Science **279**(5350): 558-60.

Han, J. W., R. B. Pearson, et al. (1995). "Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites." J Biol Chem **270**(36): 21396-403.

Hanahan, D. and J. Folkman (1996). "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis." Cell **86**(3): 353-64.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanif, I. M., M. A. Shazib, et al. (2010). "Casein Kinase II: an attractive target for anti-cancer drug design." Int J Biochem Cell Biol **42**(10): 1602-5.

Hao, Y., T. Creson, et al. (2004). "Mood stabilizer valproate promotes ERK pathway-dependent cortical neuronal growth and neurogenesis." J Neurosci **24**(29): 6590-9.

Hara, K., K. Yonezawa, et al. (1998). "Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism." J Biol Chem **273**(23): 14484-94.

Haraguchi, N., T. Utsunomiya, et al. (2006). "Characterization of a side population of cancer cells from human gastrointestinal system." Stem Cells **24**(3): 506-13.

Hardie, D. G., S. A. Hawley, et al. (2006). "AMP-activated protein kinase--development of the energy sensor concept." J Physiol **574**(Pt 1): 7-15.

Hargrave, D. (2009). "Paediatric high and low grade glioma: the impact of tumour biology on current and future therapy." Br J Neurosurg **23**(4): 351-63.

Harhaji-Trajkovic, L., U. Vilimanovich, et al. (2009). "AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells." J Cell Mol Med **13**(9B): 3644-54.

Harper, L. J., K. Piper, et al. (2007). "Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma." J Oral Pathol Med **36**(10): 594-603.

Harrington, L. S., G. M. Findlay, et al. (2004). "The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins." J Cell Biol **166**(2): 213-23.

Harris, C. C. (1996). "p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology, and cancer risk assessment." Environ Health Perspect **104 Suppl 3**: 435-9.

Hartford, C. M., A. A. Desai, et al. (2009). "A phase I trial to determine the safety, tolerability, and maximum tolerated dose of deforolimus in patients with advanced malignancies." Clin Cancer Res **15**(4): 1428-34.

Harvey, H. J., E. G. MacEwen, et al. (1977). "Neurotoxicosis associated with use of 5-fluorouracil in five dogs and one cat." J Am Vet Med Assoc **171**(3): 277-8.

Hata, A. (2001). "TGFbeta signaling and cancer." Exp Cell Res **264**(1): 111-6.

Hatzivassiliou, G., F. Zhao, et al. (2005). "ATP citrate lyase inhibition can suppress tumor cell growth." Cancer Cell **8**(4): 311-21.

Hayashi, K. (1991). "PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA." PCR Methods Appl **1**(1): 34-8.

Hayden, M. S. and S. Ghosh (2012). "NF-kappaB, the first quarter-century: remarkable progress and outstanding questions." Genes Dev **26**(3): 203-34.

He, C. and D. J. Klionsky (2009). "Regulation mechanisms and signaling pathways of autophagy." Annu Rev Genet **43**: 67-93.

Heddleston, J. M., Z. Li, et al. (2009). "The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype." Cell Cycle **8**(20): 3274-84.

Heinonen, H., A. Nieminen, et al. (2008). "Deciphering downstream gene targets of PI3K/mTOR/p70S6K pathway in breast cancer." BMC Genomics **9**: 348.

Hennessy, B. T., Y. Lu, et al. (2007). "Pharmacodynamic markers of perifosine efficacy." Clin Cancer Res **13**(24): 7421-31.

Henske, E. P., B. W. Scheithauer, et al. (1996). "Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions." Am J Hum Genet **59**(2): 400-6.

Heppner, G. H. (1984). "Tumor heterogeneity." Cancer Res **44**(6): 2259-65.

Herdegen, T., P. Skene, et al. (1997). "The c-Jun transcription factor--bipotential mediator of neuronal death, survival and regeneration." Trends Neurosci **20**(5): 227-31.

Herrington, J. and C. Carter-Su (2001). "Signaling pathways activated by the growth hormone receptor." Trends Endocrinol Metab **12**(6): 252-7.

Hershey, J. W. (2010). "Regulation of protein synthesis and the role of eIF3 in cancer." Braz J Med Biol Res **43**(10): 920-30.

Hess, G., R. Herbrecht, et al. (2009). "Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma." J Clin Oncol **27**(23): 3822-9.

Hess, J., P. Angel, et al. (2004). "AP-1 subunits: quarrel and harmony among siblings." J Cell Sci **117**(Pt 25): 5965-73.

Hirai, H., H. Sootome, et al. (2010). "MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo." Mol Cancer Ther **9**(7): 1956-67.

Hirota, S., K. Isozaki, et al. (1998). "Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors." Science **279**(5350): 577-80.

Hirschmann-Jax, C., A. E. Foster, et al. (2004). "A distinct "side population" of cells with high drug efflux capacity in human tumor cells." Proc Natl Acad Sci U S A **101**(39): 14228-33.

Hisada, M., J. E. Garber, et al. (1998). "Multiple primary cancers in families with Li-Fraumeni syndrome." J Natl Cancer Inst **90**(8): 606-11.

Ho, K. K., S. S. Myatt, et al. (2008). "Many forks in the path: cycling with FoxO." Oncogene **27**(16): 2300-11.

Ho, M. M., A. V. Ng, et al. (2007). "Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells." Cancer Res **67**(10): 4827-33.

Hoffman, K., F. A. Holmes, et al. (1996). "Phase I-II study: triciribine (tricyclic nucleoside phosphate) for metastatic breast cancer." Cancer Chemother Pharmacol **37**(3): 254-8.

Holleran, J. L., M. J. Egorin, et al. (2003). "Use of high-performance liquid chromatography to characterize the rapid decomposition of wortmannin in tissue culture media." Anal Biochem **323**(1): 19-25.

Holmen, S. L. and B. O. Williams (2005). "Essential role for Ras signaling in glioblastoma maintenance." Cancer Res **65**(18): 8250-5.

Holz, M. K. and J. Blenis (2005). "Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase." J Biol Chem **280**(28): 26089-93.

Hong, X., F. Jiang, et al. (2007). "Decrease of endogenous vascular endothelial growth factor may not affect glioma cell proliferation and invasion." J Exp Ther Oncol **6**(3): 219-29.

Horowitz, J. M., S. H. Park, et al. (1990). "Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells." Proc Natl Acad Sci U S A **87**(7): 2775-9.

Hovinga, K. E., F. Shimizu, et al. (2010). "Inhibition of notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate." Stem Cells **28**(6): 1019-29.

Howard, B. M., D. B. Gursel, et al. (2010). "EGFR signaling is differentially activated in patient-derived glioblastoma stem cells." J Exp Ther Oncol **8**(3): 247-60.

Howe, A. K. (2004). "Regulation of actin-based cell migration by cAMP/PKA." Biochim Biophys Acta **1692**(2-3): 159-74.

Howes, A. L., G. G. Chiang, et al. (2007). "The phosphatidylinositol 3-kinase inhibitor, PX-866, is a potent inhibitor of cancer cell motility and growth in three-dimensional cultures." Mol Cancer Ther **6**(9): 2505-14.

Hsieh, D., A. Hsieh, et al. (2010). "IGFBP2 promotes glioma tumor stem cell expansion and survival." Biochem Biophys Res Commun **397**(2): 367-72.

Hsing, S., Z. Gatmaitan, et al. (1992). "The function of Gp170, the multidrug-resistance gene product, in the brush border of rat intestinal mucosa." Gastroenterology **102**(3): 879-85.

Hsu, C. C., Y. W. Leu, et al. (2011). "Functional characterization of Trip10 in cancer cell growth and survival." J Biomed Sci **18**: 12.

Hu, L., J. Hofmann, et al. (2002). "Inhibition of phosphatidylinositol 3'-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer models." Cancer Res **62**(4): 1087-92.

Hu, L., C. Zaloudek, et al. (2000). "In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002)." Clin Cancer Res **6**(3): 880-6.

Huang, E. J. and L. F. Reichardt (2003). "Trk receptors: roles in neuronal signal transduction." Annu Rev Biochem **72**: 609-42.

Huang, H., S. Colella, et al. (2000). "Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays." Cancer Res **60**(24): 6868-74.

Huang, J., C. C. Dibble, et al. (2008). "The TSC1-TSC2 complex is required for proper activation of mTOR complex 2." Mol Cell Biol **28**(12): 4104-15.

Huang, S., M. A. Bjornsti, et al. (2003). "Rapamycins: mechanism of action and cellular resistance." Cancer Biol Ther **2**(3): 222-32.

Huang, S. and P. J. Houghton (2003). "Targeting mTOR signaling for cancer therapy." Curr Opin Pharmacol **3**(4): 371-7.

Hudes, G., M. Carducci, et al. (2007). "Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma." N Engl J Med **356**(22): 2271-81.

Humphrey, P. A., A. J. Wong, et al. (1988). "Amplification and expression of the epidermal growth factor receptor gene in human glioma xenografts." Cancer Res **48**(8): 2231-8.

Hunter, R. W., C. Mackintosh, et al. (2009). "Protein kinase C-mediated phosphorylation and activation of PDE3A regulate cAMP levels in human platelets." J Biol Chem **284**(18): 12339-48.

Hunter, S., E. A. Burton, et al. (1999). "Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase." J Biol Chem **274**(4): 2097-106.

Hur, E. M. and F. Q. Zhou (2010). "GSK3 signalling in neural development." Nat Rev Neurosci **11**(8): 539-51.

Huse, J. T. and E. C. Holland (2010). "Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma." Nat Rev Cancer **10**(5): 319-31.

Iadevaia, V., S. Caldarola, et al. (2008). "All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs." RNA **14**(9): 1730-6.

IARC-Working-group (2000). "IARC Working group on the evaluation of carcinogenic risks to humans: ionizing radiation, Part I, X- and gamma- radiation and neutrons. Lyon, France, 26 May-2 June 1999." IARC Monogr Eval Carcinog Risks Hum **75 Pt 1**: 1-448.

Ihle, N. T., R. Lemos, Jr., et al. (2009). "Mutations in the phosphatidylinositol-3-kinase pathway predict for antitumor activity of the inhibitor PX-866 whereas oncogenic Ras is a dominant predictor for resistance." Cancer Res **69**(1): 143-50.

Ihle, N. T., R. Williams, et al. (2004). "Molecular pharmacology and antitumor activity of PX-866, a novel inhibitor of phosphoinositide-3-kinase signaling." Mol Cancer Ther **3**(7): 763-72.

Ikenoue, T., K. Inoki, et al. (2008). "Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling." Embo J **27**(14): 1919-31.

Imamura, O., Y. Satoh, et al. (2008). "Analysis of extracellular signal-regulated kinase 2 function in neural stem/progenitor cells via nervous system-specific gene disruption." Stem Cells **26**(12): 3247-56.

Inoki, K., T. Zhu, et al. (2003). "TSC2 mediates cellular energy response to control cell growth and survival." Cell **115**(5): 577-90.

Inomata, M., S. Takahashi, et al. (2006). "Inverse correlation between Flt3 and PU.1 expression in acute myeloblastic leukemias." Leuk Res **30**(6): 659-64.

Irie, H. Y., R. V. Pearline, et al. (2005). "Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition." J Cell Biol **171**(6): 1023-34.

Islam, O., T. X. Loo, et al. (2009). "Brain-derived neurotrophic factor (BDNF) has proliferative effects on neural stem cells through the truncated TRK-B receptor, MAP kinase, AKT, and STAT-3 signaling pathways." Curr Neurovasc Res **6**(1): 42-53.

Iso, T., L. Kedes, et al. (2003). "HES and HERP families: multiple effectors of the Notch signaling pathway." J Cell Physiol **194**(3): 237-55.

Ito, K., R. Bernardi, et al. (2008). "PML targeting eradicates quiescent leukaemia-initiating cells." Nature **453**(7198): 1072-8.

Iwatani, H., T. Ito, et al. (2004). "Hematopoietic and nonhematopoietic potentials of Hoechst(low)/side population cells isolated from adult rat kidney." Kidney Int **65**(5): 1604-14.

Izzard, R. A., S. P. Jackson, et al. (1999). "Competitive and noncompetitive inhibition of the DNA-dependent protein kinase." Cancer Res **59**(11): 2581-6.

Jaafar, R., C. Zeiller, et al. (2011). "Phospholipase D regulates myogenic differentiation through the activation of both mTORC1 and mTORC2 complexes." J Biol Chem **286**(25): 22609-21.

Jackson, K. A., S. M. Majka, et al. (2001). "Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells." J Clin Invest **107**(11): 1395-402.

Jackson, K. A., T. Mi, et al. (1999). "Hematopoietic potential of stem cells isolated from murine skeletal muscle." Proc Natl Acad Sci U S A **96**(25): 14482-6.

Jackson, S. P., S. M. Schoenwaelder, et al. (2005). "PI 3-kinase p110beta: a new target for antithrombotic therapy." Nat Med **11**(5): 507-14.

Jackson, T. R., B. G. Kearns, et al. (2000). "Cytohesins and centaurins: mediators of PI 3-kinase-regulated Arf signaling." Trends Biochem Sci **25**(10): 489-95.

Jain, S. K., W. Y. Langdon, et al. (1997). "Tyrosine phosphorylation of p120cbl in BCR/abl transformed hematopoietic cells mediates enhanced association with phosphatidylinositol 3-kinase." Oncogene **14**(18): 2217-28.

Janes, M. R., J. J. Limon, et al. (2010). "Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor." Nat Med **16**(2): 205-13.

Janssens, V. and J. Goris (2001). "Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling." Biochem J **353**(Pt 3): 417-39.

Janzarik, W. G., C. P. Kratz, et al. (2007). "Further evidence for a somatic KRAS mutation in a pilocytic astrocytoma." Neuropediatrics **38**(2): 61-3.

Javelaud, D., V. I. Alexaki, et al. (2011). "TGF-beta/SMAD/GLI2 signaling axis in cancer progression and metastasis." Cancer Res **71**(17): 5606-10.

Jefferies, H. B., S. Fumagalli, et al. (1997). "Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k." Embo J **16**(12): 3693-704.

Jewell, J. L., E. Oh, et al. (2010). "Exocytosis mechanisms underlying insulin release and glucose uptake: conserved roles for Munc18c and syntaxin 4." Am J Physiol Regul Integr Comp Physiol **298**(3): R517-31.

Jhanwar-Uniyal, M., L. Albert, et al. (2011). "Deciphering the signaling pathways of cancer stem cells of glioblastoma multiforme: role of Akt/mTOR and MAPK pathways." Adv Enzyme Regul **51**(1): 164-70.

Ji, H., J. Wang, et al. (2009). "EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-Catenin from beta-Catenin and transactivation of beta-Catenin." Mol Cell **36**(4): 547-59.

Jia, S., Z. Liu, et al. (2008). "Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis." Nature **454**(7205): 776-9.

Jiang, X. and X. Wang (2004). "Cytochrome C-mediated apoptosis." Annu Rev Biochem **73**: 87-106.

Jiang, Y., B. N. Jahagirdar, et al. (2002). "Pluripotency of mesenchymal stem cells derived from adult marrow." Nature **418**(6893): 41-9.

Jin, L., K. J. Hope, et al. (2006). "Targeting of CD44 eradicates human acute myeloid leukemic stem cells." Nat Med **12**(10): 1167-74.

Jin, L., E. M. Lee, et al. (2009). "Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells." Cell Stem Cell **5**(1): 31-42.

Johnson, L. N., M. E. Noble, et al. (1996). "Active and inactive protein kinases: structural basis for regulation." Cell **85**(2): 149-58.

Jordan, C. T., D. Upchurch, et al. (2000). "The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells." Leukemia **14**(10): 1777-84.

Julien, L. A., A. Carriere, et al. (2010). "mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling." Mol Cell Biol **30**(4): 908-21.

Jung, C. H., S. H. Ro, et al. (2010). "mTOR regulation of autophagy." FEBS Lett **584**(7): 1287-95.

Jungmann, R. A. and O. Kiryukhina (2005). "Cyclic AMP and AKAP-mediated targeting of protein kinase A regulates lactate dehydrogenase subunit A mRNA stability." J Biol Chem **280**(26): 25170-7.

Kaibuchi, K., S. Kuroda, et al. (1999). "Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells." Annu Rev Biochem **68**: 459-86.

Kaiser, U., B. Auerbach, et al. (1996). "The neural cell adhesion molecule NCAM in multiple myeloma." Leuk Lymphoma **20**(5-6): 389-95.

Kambara, H., H. Okano, et al. (2005). "An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor." Cancer Res **65**(7): 2832-9.

Kamimoto, Y., Z. Gatmaitan, et al. (1989). "The function of Gp170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles." J Biol Chem **264**(20): 11693-8.

Kaminska, B., A. Ellert-Miklaszewska, et al. (2009). "Efficacy and mechanism of anti-tumor action of new potential CK2 inhibitors toward glioblastoma cells." Int J Oncol **35**(5): 1091-100.

Kamura, S., Y. Matsumoto, et al. (2010). "Basic fibroblast growth factor in the bone microenvironment enhances cell motility and invasion of Ewing's sarcoma family of tumours by activating the FGFR1-PI3K-Rac1 pathway." Br J Cancer **103**(3): 370-81.

Kang, C. B., Y. Hong, et al. (2008). "FKBP family proteins: immunophilins with versatile biological functions." Neurosignals **16**(4): 318-25.

Kapeller, R., K. V. Prasad, et al. (1994). "Identification of two SH3-binding motifs in the regulatory subunit of phosphatidylinositol 3-kinase." J Biol Chem **269**(3): 1927-33.

Kapoor, G. S. and D. M. O'Rourke (2010). "SIRPalpha1 receptors interfere with the EGFRvIII signalosome to inhibit glioblastoma cell transformation and migration." Oncogene **29**(29): 4130-44.

Kapoor, G. S., Y. Zhan, et al. (2004). "Distinct domains in the SHP-2 phosphatase differentially regulate epidermal growth factor receptor/NF-kappaB activation through Gab1 in glioblastoma cells." Mol Cell Biol **24**(2): 823-36.

Kario, E., M. D. Marmor, et al. (2005). "Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling." J Biol Chem **280**(8): 7038-48.

Karlsson, E., M. A. Waltersson, et al. (2010). "High-resolution genomic analysis of the 11q13 amplicon in breast cancers identifies synergy with 8p12 amplification, involving the mTOR targets S6K2 and 4EBP1." Genes Chromosomes Cancer **50**(10): 775-87.

Karsy, M., L. Albert, et al. (2010). "All-trans retinoic acid modulates cancer stem cells of glioblastoma multiforme in an MAPK-dependent manner." Anticancer Res **30**(12): 4915-20.

Kashiwaba, M., K. Katsura, et al. (2003). "A novel protein phosphatase 2C family member (PP2Czeta) is able to associate with ubiquitin conjugating enzyme 9." FEBS Lett **538**(1-3): 197-202.

Katayama, K., N. Fujita, et al. (2005). "Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle progression at G2/M transition." Mol Cell Biol **25**(13): 5725-37.

Kato, K., T. Yazawa, et al. (2012). "The inositol 5-phosphatase SHIP2 is an effector of RhoA and is involved in cell polarity and migration." Mol Biol Cell.

Katoh, M. (2007). "Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis." Stem Cell Rev **3**(1): 30-8.

Katoh, M. (2007). "WNT signaling pathway and stem cell signaling network." Clin Cancer Res **13**(14): 4042-5.

Kaur, S., K. Leszczynska, et al. (2011). "RhoJ/TCL regulates endothelial motility and tube formation and modulates actomyosin contractility and focal adhesion numbers." Arterioscler Thromb Vasc Biol **31**(3): 657-64.

Kawamata, T., T. Taniguchi, et al. (1998). "A protein kinase, PKN, accumulates in Alzheimer neurofibrillary tangles and associated endoplasmic reticulum-derived vesicles and phosphorylates tau protein." J Neurosci **18**(18): 7402-10.

Kawata, T., H. Ito, et al. (2004). "G2 chromatid damage and repair kinetics in normal human fibroblast cells exposed to low- or high-LET radiation." Cytogenet Genome Res **104**(1-4): 211-5.

Kelly, K. J., R. M. Sandoval, et al. (2003). "A novel method to determine specificity and sensitivity of the TUNEL reaction in the quantitation of apoptosis." Am J Physiol Cell Physiol **284**(5): C1309-18.

Kelly, P. N., A. Dakic, et al. (2007). "Tumor growth need not be driven by rare cancer stem cells." Science **317**(5836): 337.

Kelsall, I. R., M. Voss, et al. (2011). "R3F, a novel membrane-associated glycogen targeting subunit of protein phosphatase 1 regulates glycogen synthase in astrocytoma cells in response to glucose and extracellular signals." J Neurochem **118**(4): 596-610.

Kent, M. S., C. J. Collins, et al. (2009). "Activation of the AKT and mammalian target of rapamycin pathways and the inhibitory effects of rapamycin on those pathways in canine malignant melanoma cell lines." Am J Vet Res **70**(2): 263-9.

Khaleghpour, K., A. Kahvejian, et al. (2001). "Dual interactions of the translational repressor Paip2 with poly(A) binding protein." Mol Cell Biol **21**(15): 5200-13.

Khalil, B. D. and M. El-Sibai (2012). "Rho GTPases in primary brain tumor malignancy and invasion." J Neurooncol.

Kharas, M. G., R. Okabe, et al. (2010). "Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice." Blood **115**(7): 1406-15.

Khatua, S., K. M. Peterson, et al. (2003). "Overexpression of the EGFR/FKBP12/HIF-2alpha pathway identified in childhood astrocytomas by angiogenesis gene profiling." Cancer Res **63**(8): 1865-70.

Kim, K. J., B. Li, et al. (1993). "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo." Nature **362**(6423): 841-4.

Kim, M. and C. M. Morshead (2003). "Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis." J Neurosci **23**(33): 10703-9.

Kim, M. J., R. K. Kim, et al. (2011). "Importance of PKCdelta signaling in fractionated-radiation-induced expansion of glioma-initiating cells and resistance to cancer treatment." J Cell Sci **124**(Pt 18): 3084-94.

Kim, M. S., E. G. Jeong, et al. (2008). "Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias." Br J Cancer **98**(9): 1533-5.

Kim, S., J. Hwang, et al. (2008). "Role of protein kinase Cdelta in paraquat-induced glial cell death." J Neurosci Res **86**(9): 2062-70.

Kim, Y. M., T. Watanabe, et al. (2003). "PNUTS, a protein phosphatase 1 (PP1) nuclear targeting subunit. Characterization of its PP1- and RNA-binding domains and regulation by phosphorylation." J Biol Chem **278**(16): 13819-28.

Kimmelman, A. C., N. Nunez Rodriguez, et al. (2002). "R-Ras3/M-Ras induces neuronal differentiation of PC12 cells through cell-type-specific activation of the mitogen-activated protein kinase cascade." Mol Cell Biol **22**(16): 5946-61.

Kimmelman, A. C., M. Osada, et al. (2000). "R-Ras3, a brain-specific Ras-related protein, activates Akt and promotes cell survival in PC12 cells." Oncogene **19**(16): 2014-22.

Kinzler, K. W. and B. Vogelstein (1996). "Lessons from hereditary colorectal cancer." Cell **87**(2): 159-70.

Kirkegaard, T., C. J. Witton, et al. (2010). "Molecular alterations in AKT1, AKT2 and AKT3 detected in breast and prostatic cancer by FISH." Histopathology **56**(2): 203-11.

Klopotowska, D. and L. Strzadala (2005). "[The role of TrkC receptor and neurotrophin 3 in the development and function of neural cells]." Postepy Hig Med Dosw (Online) **59**: 517-22.

Knapp, D. W., R. C. Richardson, et al. (1987). "Cisplatin toxicity in cats." J Vet Intern Med **1**(1): 29-35.

Knizetova, P., J. Ehrmann, et al. (2008). "Autocrine regulation of glioblastoma cell cycle progression, viability and radioresistance through the VEGF-VEGFR2 (KDR) interplay." Cell Cycle **7**(16): 2553-61.

Knobbe, C. B. and G. Reifenberger (2003). "Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas." Brain Pathol **13**(4): 507-18.

Knobbe, C. B., J. Reifenberger, et al. (2004). "Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas." J Natl Cancer Inst **96**(6): 483-6.

Knobbe, C. B., J. Reifenberger, et al. (2004). "Mutation analysis of the Ras pathway genes NRAS, HRAS, KRAS and BRAF in glioblastomas." Acta Neuropathol **108**(6): 467-70.

Kondapaka, S. B., S. S. Singh, et al. (2003). "Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation." Mol Cancer Ther **2**(11): 1093-103.

Kondo, T., T. Setoguchi, et al. (2004). "Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line." Proc Natl Acad Sci U S A **101**(3): 781-6.

Konduri, S. and R. E. Schwarz (2007). "Estrogen receptor beta/alpha ratio predicts response of pancreatic cancer cells to estrogens and phytoestrogens." J Surg Res **140**(1): 55-66.

Kong, D., S. Dan, et al. (2010). "Inhibition profiles of phosphatidylinositol 3-kinase inhibitors against PI3K superfamily and human cancer cell line panel JFCR39." Eur J Cancer **46**(6): 1111-21.

Kong, D., S. Yaguchi, et al. (2009). "Effect of ZSTK474, a novel phosphatidylinositol 3-kinase inhibitor, on DNA-dependent protein kinase." Biol Pharm Bull **32**(2): 297-300.

Kong, D. and T. Yamori (2007). "ZSTK474 is an ATP-competitive inhibitor of class I phosphatidylinositol 3 kinase isoforms." Cancer Sci **98**(10): 1638-42.

Kong, D. and T. Yamori (2008). "Phosphatidylinositol 3-kinase inhibitors: promising drug candidates for cancer therapy." Cancer Sci **99**(9): 1734-40.

Koromilas, A. E., A. Lazaris-Karatzas, et al. (1992). "mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E." Embo J **11**(11): 4153-8.

Korsten, H., A. Ziel-van der Made, et al. (2009). "Accumulating progenitor cells in the luminal epithelial cell layer are candidate tumor initiating cells in a Pten knockout mouse prostate cancer model." PLoS One **4**(5): e5662.

Korur, S., R. M. Huber, et al. (2009). "GSK3beta regulates differentiation and growth arrest in glioblastoma." PLoS One **4**(10): e7443.

Koul, D., R. Shen, et al. (2006). "Inhibition of Akt survival pathway by a small-molecule inhibitor in human glioblastoma." Mol Cancer Ther **5**(3): 637-44.

Koul, D., R. Shen, et al. (2010). "Cellular and in vivo activity of a novel PI3K inhibitor, PX-866, against human glioblastoma." Neuro Oncol **12**(6): 559-69.

Koul, D., R. Shen, et al. (2007). "PTEN down regulates AP-1 and targets c-fos in human glioma cells via PI3-kinase/Akt pathway." Mol Cell Biochem **300**(1-2): 77-87.

Kouri, F. M., S. A. Jensen, et al. (2012). "The role of Bcl-2 family proteins in therapy responses of malignant astrocytic gliomas: Bcl2L12 and beyond." ScientificWorldJournal **2012**: 838916.

Kraegel, S. A., P. H. Gumerlock, et al. (1992). "K-ras activation in non-small cell lung cancer in the dog." Cancer Res **52**(17): 4724-7.

Krugmann, S., K. E. Anderson, et al. (2002). "Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices." Mol Cell **9**(1): 95-108.

Krysko, D. V., T. Vanden Berghe, et al. (2008). "Apoptosis and necrosis: detection, discrimination and phagocytosis." Methods **44**(3): 205-21.

Kubota, Y. (2012). "Tumor angiogenesis and anti-angiogenic therapy." Keio J Med **61**(2): 47-56.

Kumar, C. C. and V. Madison (2005). "AKT crystal structure and AKT-specific inhibitors." Oncogene **24**(50): 7493-501.

Kuper, H., H. O. Adami, et al. (2000). "Infections as a major preventable cause of human cancer." J Intern Med **248**(3): 171-83.

Kuratomi, G., A. Komuro, et al. (2005). "NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor." Biochem J **386**(Pt 3): 461-70.

Kurig, B., A. Shymanets, et al. (2009). "Ras is an indispensable coregulator of the class IB phosphoinositide 3-kinase p87/p110gamma." Proc Natl Acad Sci U S A **106**(48): 20312-7.

Kurzrock, R., H. M. Kantarjian, et al. (2003). "Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics." Ann Intern Med **138**(10): 819-30.

Kwitkowski, V. E., T. M. Prowell, et al. (2010). "FDA approval summary: temsirolimus as treatment for advanced renal cell carcinoma." Oncologist **15**(4): 428-35.

Lamszus, K. and H. S. Gunther (2010). "Glioma stem cells as a target for treatment." Target Oncol **5**(3): 211-5.

Lane, D. P. and S. Benchimol (1990). "p53: oncogene or anti-oncogene?" Genes Dev **4**(1): 1-8.

Lankas, G. R., L. D. Wise, et al. (1998). "Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice." Reprod Toxicol **12**(4): 457-63.

Larocca, C. and J. Schlom (2011). "Viral vector-based therapeutic cancer vaccines." Cancer J **17**(5): 359-71.

Lathia, J. D., J. Gallagher, et al. (2010). "Integrin alpha 6 regulates glioblastoma stem cells." Cell Stem Cell **6**(5): 421-32.

Le Mercier, M., F. Lefranc, et al. (2008). "Evidence of galectin-1 involvement in glioma chemoresistance." Toxicol Appl Pharmacol **229**(2): 172-83.

Lebedeva, I. V., P. Pande, et al. (2011). "Sensitive and specific fluorescent probes for functional analysis of the three major types of mammalian ABC transporters." PLoS One **6**(7): e22429.

Lee, A., J. D. Kessler, et al. (2005). "Isolation of neural stem cells from the postnatal cerebellum." Nat Neurosci **8**(6): 723-9.

Lee, C. J., J. Dosch, et al. (2008). "Pancreatic cancer stem cells." J Clin Oncol **26**(17): 2806-12.

Lee, D. F., H. P. Kuo, et al. (2007). "IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway." Cell **130**(3): 440-55.

Lee, H. and A. Y. Tsygankov (2010). "c-Cbl regulates glioma invasion through matrix metalloproteinase 2." J Cell Biochem **111**(5): 1169-78.

Lee, J., M. J. Son, et al. (2008). "Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells." Cancer Cell **13**(1): 69-80.

Lee, J. T., Jr., L. S. Steelman, et al. (2004). "Phosphatidylinositol 3'-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells." Cancer Res **64**(22): 8397-404.

Lee, M. N., S. H. Ha, et al. (2009). "Glycolytic flux signals to mTOR through glyceraldehyde-3-phosphate dehydrogenase-mediated regulation of Rheb." Mol Cell Biol **29**(14): 3991-4001.

Lee, M. W., D. S. Kim, et al. (2011). "The roles of AKT1 and AKT2 in non-small cell lung cancer cell survival, growth and migration." Cancer Sci.

Lemmon, M. A. (2003). "Phosphoinositide recognition domains." Traffic **4**(4): 201-13.

Lemmon, M. A. and J. Schlessinger (2010). "Cell signaling by receptor tyrosine kinases." Cell **141**(7): 1117-34.

Lengauer, C., K. W. Kinzler, et al. (1998). "Genetic instabilities in human cancers." Nature **396**(6712): 643-9.

Leonard, W. J. and J. J. O'Shea (1998). "Jaks and STATs: biological implications." Annu Rev Immunol **16**: 293-322.

Levine, R. A., T. Forest, et al. (2002). "Tumor suppressor PTEN is mutated in canine osteosarcoma cell lines and tumors." Vet Pathol **39**(3): 372-8.

Levy, D. S., J. A. Kahana, et al. (2009). "AKT inhibitor, GSK690693, induces growth inhibition and apoptosis in acute lymphoblastic leukemia cell lines." Blood **113**(8): 1723-9.

LeWitt, P. A., A. R. Rezai, et al. (2011). "AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial." Lancet Neurol **10**(4): 309-19.

Li, C., D. G. Heidt, et al. (2007). "Identification of pancreatic cancer stem cells." Cancer Res **67**(3): 1030-7.

Li, X., B. Monks, et al. (2007). "Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator." Nature **447**(7147): 1012-6.

Li, X. J., Q. H. Guo, et al. (2012). "LRP16 gene protects mouse insulinoma MIN6 cells against fatty acid-induced apoptosis through Akt/FoxO1 signaling." Chin Med J (Engl) **125**(10): 1695-702.

Li, Y., K. Inoki, et al. (2003). "The p38 and MK2 kinase cascade phosphorylates tuberin, the tuberous sclerosis 2 gene product, and enhances its interaction with 14-3-3." J Biol Chem **278**(16): 13663-71.

Li, Y., H. M. Lu, et al. (2010). "Glycogen synthase kinase-3beta regulates astrocytic differentiation of U87-MG human glioblastoma cells." Acta Pharmacol Sin **31**(3): 355-60.

Li, Z., S. Bao, et al. (2009). "Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells." Cancer Cell **15**(6): 501-13.

Liang, H., X. Ding, et al. (2012). "Knockdown of eukaryotic translation initiation factors 3B (EIF3B) inhibits proliferation and promotes apoptosis in glioblastoma cells." Neurol Sci.

Liang, J., J. Zubovitz, et al. (2002). "PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest." Nat Med **8**(10): 1153-60.

Liang, S., G. Shen, et al. (2009). "Isoform-specific expression and characterization of 14-3-3 proteins in human glioma tissues discovered by stable isotope labeling with amino acids in cell culture-based proteomic analysis." Proteomics Clin Appl **3**(6): 743-53.

Licata, S., A. Saponiero, et al. (2000). "Doxorubicin metabolism and toxicity in human myocardium: role of cytoplasmic deglycosidation and carbonyl reduction." Chem Res Toxicol **13**(5): 414-20.

Lim, K. H. and C. M. Counter (2005). "Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance." Cancer Cell **8**(5): 381-92.

Lin, J., X. M. Zhang, et al. (2010). "gamma-secretase inhibitor-I enhances radiosensitivity of glioblastoma cell lines by depleting CD133+ tumor cells." Arch Med Res **41**(7): 519-29.

Lin, T. A., X. Kong, et al. (1995). "Control of PHAS-I by insulin in 3T3-L1 adipocytes. Synthesis, degradation, and phosphorylation by a rapamycin-sensitive and mitogen-activated protein kinase-independent pathway." J Biol Chem **270**(31): 18531-8.

Lin, Y., T. Jiang, et al. (2009). "Plasma IGFBP-2 levels predict clinical outcomes of patients with high-grade gliomas." Neuro Oncol **11**(5): 468-76.

Lin, Y. W., Z. M. Beharry, et al. (2010). "A small molecule inhibitor of Pim protein kinases blocks the growth of precursor T-cell lymphoblastic leukemia/lymphoma." Blood **115**(4): 824-33.

Lindsley, C. W. (2010). "The Akt/PKB family of protein kinases: a review of small molecule inhibitors and progress towards target validation: a 2009 update." Curr Top Med Chem **10**(4): 458-77.

Lindsley, C. W., S. F. Barnett, et al. (2007). "Recent progress in the development of ATP-competitive and allosteric Akt kinase inhibitors." Curr Top Med Chem **7**(14): 1349-63.

Liu, G., X. Yuan, et al. (2006). "Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma." Mol Cancer **5**: 67.

Liu, J. P. (1999). "Studies of the molecular mechanisms in the regulation of telomerase activity." FASEB J **13**(15): 2091-104.

Liu, K. W., H. Feng, et al. (2011). "SHP-2/PTPN11 mediates gliomagenesis driven by PDGFRA and INK4A/ARF aberrations in mice and humans." J Clin Invest **121**(3): 905-17.

Liu, P., H. Cheng, et al. (2009). "Targeting the phosphoinositide 3-kinase pathway in cancer." Nat Rev Drug Discov **8**(8): 627-44.

Liu, R., D. Liu, et al. (2011). "The Akt-specific inhibitor MK2206 selectively inhibits thyroid cancer cells harboring mutations that can activate the PI3K/Akt pathway." J Clin Endocrinol Metab **96**(4): E577-85.

Liu, Y., C. Li, et al. (2010). "STAT3 as a Therapeutic Target for Glioblastoma." Anticancer Agents Med Chem **10**(7): 512-9.

Liu, Z., P. Hou, et al. (2008). "Highly prevalent genetic alterations in receptor tyrosine kinases and phosphatidylinositol 3-kinase/akt and mitogen-activated protein kinase pathways in anaplastic and follicular thyroid cancers." J Clin Endocrinol Metab **93**(8): 3106-16.

Lodillinsky, C., Y. Langle, et al. (2010). "Bacillus Calmette Guerin induces fibroblast activation both directly and through macrophages in a mouse bladder cancer model." PLoS One **5**(10): e13571.

Loffing, J., S. Y. Flores, et al. (2006). "Sgk kinases and their role in epithelial transport." Annu Rev Physiol **68**: 461-90.

Lohmann, S. M., G. Schwoch, et al. (1983). "Dibutyryl cAMP treatment of neuroblastoma-glioma hybrid cells results in selective increase in cAMP-receptor protein (R-I) as measured by monospecific antibodies." Embo J **2**(2): 153-9.

Loi, S., E. de Azambuja, et al. (2011). "HER2-overexpressing breast cancer: time for the cure with less chemotherapy?" Curr Opin Oncol **23**(6): 547-58.

Lokker, N. A., C. M. Sullivan, et al. (2002). "Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors." Cancer Res **62**(13): 3729-35.

London, C. A., S. J. Galli, et al. (1999). "Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit." Exp Hematol **27**(4): 689-97.

Longley, D. B., D. P. Harkin, et al. (2003). "5-fluorouracil: mechanisms of action and clinical strategies." Nat Rev Cancer **3**(5): 330-8.

Lonn, S., N. Rothman, et al. (2008). "Genetic variation in insulin-like growth factors and brain tumor risk." Neuro Oncol **10**(4): 553-9.

Los, M., J. M. Roodhart, et al. (2007). "Target practice: lessons from phase III trials with bevacizumab and vatalanib in the treatment of advanced colorectal cancer." Oncologist **12**(4): 443-50.

Lou, X., Q. Zhou, et al. (2009). "Inhibition of the met receptor tyrosine kinase signaling enhances the chemosensitivity of glioma cell lines to CDDP through activation of p38 MAPK pathway." Mol Cancer Ther **8**(5): 1126-36.

Louis, S. A., R. L. Rietze, et al. (2008). "Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay." Stem Cells **26**(4): 988-96.

Lu, J., Z. Zhuang, et al. (2010). "The effect of a PP2A inhibitor on the nuclear receptor corepressor pathway in glioma." J Neurosurg **113**(2): 225-33.

Lu, W., S. Finnis, et al. (2007). "Tyrosine 311 is phosphorylated by c-Abl and promotes the apoptotic effect of PKCdelta in glioma cells." Biochem Biophys Res Commun **352**(2): 431-6.

Luk, S. K., R. P. Piekorz, et al. (2012). "The catalytic phosphoinositol 3-kinase isoform p110delta is required for glioma cell migration and invasion." Eur J Cancer **48**(1): 149-57.

Lyman, S. D. and S. E. Jacobsen (1998). "c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities." Blood **91**(4): 1101-34.

Lyustikman, Y., H. Momota, et al. (2008). "Constitutive activation of Raf-1 induces glioma formation in mice." Neoplasia **10**(5): 501-10.

Ma, L., J. Teruya-Feldstein, et al. (2007). "Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer." Cancer Res **67**(15): 7106-12.

Ma, S., K. W. Chan, et al. (2007). "Identification and characterization of tumorigenic liver cancer stem/progenitor cells." Gastroenterology **132**(7): 2542-56.

Ma, S., Y. Yang, et al. (2009). "Endogenous human CaMKII inhibitory protein suppresses tumor growth by inducing cell cycle arrest and apoptosis through down-regulation of the phosphatidylinositide 3-kinase/Akt/HDM2 pathway." J Biol Chem **284**(37): 24773-82.

Ma, X. M., S. O. Yoon, et al. (2008). "SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs." Cell **133**(2): 303-13.

Ma, Y., B. J. Longley, et al. (1999). "Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms." J Invest Dermatol **112**(2): 165-70.

Maccario, H., N. M. Perera, et al. (2007). "PTEN is destabilized by phosphorylation on Thr366." Biochem J **405**(3): 439-44.

Maehama, T. and J. E. Dixon (1998). "The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate." J Biol Chem **273**(22): 13375-8.

Maira, S. M., F. Stauffer, et al. (2008). "Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity." Mol Cancer Ther **7**(7): 1851-63.

Mak, B. C. and R. S. Yeung (2004). "The tuberous sclerosis complex genes in tumor development." Cancer Invest **22**(4): 588-603.

Malhotra, R., K. M. D'Souza, et al. (2010). "G alpha(q)-mediated activation of GRK2 by mechanical stretch in cardiac myocytes: the role of protein kinase C." J Biol Chem **285**(18): 13748-60.

Mamane, Y., E. Petroulakis, et al. (2004). "eIF4E--from translation to transformation." Oncogene **23**(18): 3172-9.

Manara, M. C., G. Nicoletti, et al. (2010). "NVP-BEZ235 as a new therapeutic option for sarcomas." Clin Cancer Res **16**(2): 530-40.

Mandal, M., S. Kim, et al. (2005). "The Akt inhibitor KP372-1 suppresses Akt activity and cell proliferation and induces apoptosis in thyroid cancer cells." Br J Cancer **92**(10): 1899-905.

Mandal, M., M. Younes, et al. (2006). "The Akt inhibitor KP372-1 inhibits proliferation and induces apoptosis and anoikis in squamous cell carcinoma of the head and neck." Oral Oncol **42**(4): 430-9.

Mani, S. A., W. Guo, et al. (2008). "The epithelial-mesenchymal transition generates cells with properties of stem cells." Cell **133**(4): 704-15.

Manning, B. D. and L. C. Cantley (2003). "Rheb fills a GAP between TSC and TOR." Trends Biochem Sci **28**(11): 573-6.

Manning, B. D. and L. C. Cantley (2007). "AKT/PKB signaling: navigating downstream." Cell **129**(7): 1261-74.

Manning, B. D., A. R. Tee, et al. (2002). "Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway." Mol Cell **10**(1): 151-62.

Markowitz, S., J. Wang, et al. (1995). "Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability." Science **268**(5215): 1336-8.

Marshall, C. J. (1995). "Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation." Cell **80**(2): 179-85.

Marshall, G. P., 2nd, B. A. Reynolds, et al. (2007). "Using the neurosphere assay to quantify neural stem cells in vivo." Curr Pharm Biotechnol **8**(3): 141-5.

Martelli, A. M., C. Evangelisti, et al. (2010). "The emerging role of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in normal myelopoiesis and leukemogenesis." Biochim Biophys Acta **1803**(9): 991-1002.

Martineau, Y., M. C. Derry, et al. (2008). "Poly(A)-binding protein-interacting protein 1 binds to eukaryotic translation initiation factor 3 to stimulate translation." Mol Cell Biol **28**(21): 6658-67.

Masri, J., A. Bernath, et al. (2007). "mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor." Cancer Res **67**(24): 11712-20.

Matsuda, T., T. Nakamura, et al. (1999). "STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells." Embo J **18**(15): 4261-9.

Matsumoto, K., T. Asano, et al. (1997). "Novel small GTPase M-Ras participates in reorganization of actin cytoskeleton." Oncogene **15**(20): 2409-17.

Mattoon, D. R., B. Lamothe, et al. (2004). "The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway." BMC Biol **2**: 24.

Maurer, M., T. Su, et al. (2009). "3-Phosphoinositide-dependent kinase 1 potentiates upstream lesions on the phosphatidylinositol 3-kinase pathway in breast carcinoma." Cancer Res **69**(15): 6299-306.

Maurer, U., C. Charvet, et al. (2006). "Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1." Mol Cell **21**(6): 749-60.

McDonald, G. T., R. Sullivan, et al. (2010). "Inhibition of phosphatidylinositol 3-kinase promotes tumor cell resistance to chemotherapeutic agents via a mechanism involving delay in cell cycle progression." Exp Cell Res **316**(19): 3197-206.

McLaughlin, N., B. Annabi, et al. (2006). "The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate." Brain Res **1071**(1): 1-9.

Medema, R. H., G. J. Kops, et al. (2000). "AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1." Nature **404**(6779): 782-7.

Mei, Y., Y. Zhang, et al. (2009). "FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation." Proc Natl Acad Sci U S A **106**(13): 5153-8.

Meister, G., M. Landthaler, et al. (2004). "Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs." Mol Cell **15**(2): 185-97.

Meng, J., B. Dai, et al. (2010). "Combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo." PLoS One **5**(11): e14124.

Meshinchi, S. and F. R. Appelbaum (2009). "Structural and functional alterations of FLT3 in acute myeloid leukemia." Clin Cancer Res **15**(13): 4263-9.

Messaoudi, S., J. F. Peyrat, et al. (2008). "Recent advances in Hsp90 inhibitors as antitumor agents." Anticancer Agents Med Chem **8**(7): 761-82.

Meyuhas, O. and E. Hornstein (2000). "Translational control of TOP mRNAs. ." In: Translational Control of Gene Expression (Sonenberg, N., Hershey,J.W.B. & Mathews, M. B., eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miano, J. M. (2010). "Role of serum response factor in the pathogenesis of disease." Lab Invest **90**(9): 1274-84.

Miki, T., S. Y. Yasuda, et al. (2011). "Wnt/beta-catenin signaling in embryonic stem cell self-renewal and somatic cell reprogramming." Stem Cell Rev **7**(4): 836-46.

Miller, F. R., B. E. Miller, et al. (1983). "Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability." Invasion Metastasis **3**(1): 22-31.

Misawa, H., M. Ohtsubo, et al. (1998). "Cloning and characterization of a novel class II phosphoinositide 3-kinase containing C2 domain." Biochem Biophys Res Commun **244**(2): 531-9.

Mishra, A., Y. Shiozawa, et al. (2011). "Homing of Cancer Cells to the Bone." Cancer Microenviron **4**(3): 221-35.

Misirkic, M., K. Janjetovic, et al. (2012). "Inhibition of AMPK-dependent autophagy enhances in vitro antiglioma effect of simvastatin." Pharmacol Res **65**(1): 111-9.

Mita, M., K. Sankhala, et al. (2008). "Deforolimus (AP23573) a novel mTOR inhibitor in clinical development." Expert Opin Investig Drugs **17**(12): 1947-54.

Mitsutake, N., A. Iwao, et al. (2007). "Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively." Endocrinology **148**(4): 1797-803.

Mittelman, A., E. S. Casper, et al. (1983). "Phase I study of tricyclic nucleoside phosphate." Cancer Treat Rep **67**(2): 159-62.

Miyamoto, S., H. Teramoto, et al. (1995). "Integrin function: molecular hierarchies of cytoskeletal and signaling molecules." J Cell Biol **131**(3): 791-805.

Miyashita, K., K. Kawakami, et al. (2009). "Potential therapeutic effect of glycogen synthase kinase 3beta inhibition against human glioblastoma." Clin Cancer Res **15**(3): 887-97.

Miyauchi, T., M. Miyata, et al. (2012). "Waon Therapy Upregulates Hsp90 and Leads to Angiogenesis Through the Akt-Endothelial Nitric Oxide Synthase Pathway in Mouse Hindlimb Ischemia." Circ J.

Miyazono, K., S. Ehata, et al. (2012). "Tumor-promoting functions of transforming growth factor-beta in progression of cancer." Ups J Med Sci **117**(2): 143-52.

Mizoguchi, M., C. L. Nutt, et al. (2004). "Genetic alterations of phosphoinositide 3-kinase subunit genes in human glioblastomas." Brain Pathol **14**(4): 372-7.

Mizrak, D., M. Brittan, et al. (2008). "CD133: molecule of the moment." J Pathol **214**(1): 3-9.

Modur, V., R. Nagarajan, et al. (2002). "FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer." J Biol Chem **277**(49): 47928-37.

Mohammad-Qureshi, S. S., M. D. Jennings, et al. (2008). "Clues to the mechanism of action of eIF2B, the guanine-nucleotide-exchange factor for translation initiation." Biochem Soc Trans **36**(Pt 4): 658-64.

Mohn, F., M. Weber, et al. (2009). "Methylated DNA immunoprecipitation (MeDIP)." Methods Mol Biol **507**: 55-64.

Mole, D., T. Gagliano, et al. (2011). "Targeting protein kinase C by enzastaurin restrains proliferation and secretion in human pancreatic endocrine tumors." Endocr Relat Cancer.

Momparler, R. L., M. Karon, et al. (1976). "Effect of adriamycin on DNA, RNA, and protein synthesis in cell-free systems and intact cells." Cancer Res **36**(8): 2891-5.

Moodie, S. A., B. M. Willumsen, et al. (1993). "Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase." Science **260**(5114): 1658-61.

Moon, B. S., J. Y. Yoon, et al. (2009). "Bone morphogenetic protein 4 stimulates neuronal differentiation of neuronal stem cells through the ERK pathway." Exp Mol Med **41**(2): 116-25.

Moon, J. S., W. J. Jin, et al. (2011). "Androgen stimulates glycolysis for de novo lipid synthesis by increasing the activities of hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 in prostate cancer cells." Biochem J **433**(1): 225-33.

Moore, A. S. and A. E. Frimberger (2000). Principles of chemotherapy. Textbook of veterinary internal medicine: diseases of the dog and cat. S. J. Ettinger and E. C. Feldman. Philadelphia, Pennsylvania, USA, W.B. Saunders Company. **1:** 484-89.

Moreno, M. J., M. Ball, et al. (2006). "Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells." Glia **53**(8): 845-57.

Morrison, S. J., S. E. Perez, et al. (2000). "Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells." Cell **101**(5): 499-510.

Morrison, W. B. (1979). "Paraneoplastic syndromes of the dog." J Am Vet Med Assoc **175**(6): 559-61.

Mothe-Satney, I., G. J. Brunn, et al. (2000). "Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies." J Biol Chem **275**(43): 33836-43.

Motzer, R. J., B. Escudier, et al. (2010). "Phase 3 trial of everolimus for metastatic renal cell carcinoma : final results and analysis of prognostic factors." Cancer **116**(18): 4256-65.

Motzer, R. J., B. Escudier, et al. (2008). "Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial." Lancet **372**(9637): 449-56.

Moulton, J. E. (1990). Tumours in dosmetic animals. Berkeley, University of California Press.

Mulholland, P. J., H. Fiegler, et al. (2006). "Genomic profiling identifies discrete deletions associated with translocations in glioblastoma multiforme." Cell Cycle **5**(7): 783-91.

Mullighan, C. G., L. A. Phillips, et al. (2008). "Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia." Science **322**(5906): 1377-80.

Munger, K., B. A. Werness, et al. (1989). "Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product." Embo J **8**(13): 4099-105.

Murakami, M. and M. Simons (2008). "Fibroblast growth factor regulation of neovascularization." Curr Opin Hematol **15**(3): 215-20.

Mure, H., K. Matsuzaki, et al. (2010). "Akt2 and Akt3 play a pivotal role in malignant gliomas." Neuro Oncol **12**(3): 221-32.

Nagano, T., Y. Yamada, et al. (2007). "Docetaxel: a therapeutic option in the treatment of cutaneous angiosarcoma: report of 9 patients." Cancer **110**(3): 648-51.

Nagasawa, T., Y. Omatsu, et al. (2011). "Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells." Trends Immunol **32**(7): 315-20.

Nagasawa, T., K. Tachibana, et al. (1998). "A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection." Semin Immunol **10**(3): 179-85.

Nager, M., D. Bhardwaj, et al. (2012). "beta-Catenin Signalling in Glioblastoma Multiforme and Glioma-Initiating Cells." Chemother Res Pract **2012**: 192362.

Nakada, M., K. L. Drake, et al. (2006). "Ephrin-B3 ligand promotes glioma invasion through activation of Rac1." Cancer Res **66**(17): 8492-500.

Nakae, J., T. Kitamura, et al. (2001). "The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression." J Clin Invest **108**(9): 1359-67.

Nakanishi, S., K. J. Catt, et al. (1995). "A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids." Proc Natl Acad Sci U S A **92**(12): 5317-21.

Nakanishi, S., H. Yano, et al. (1995). "Novel functions of phosphatidylinositol 3-kinase in terminally differentiated cells." Cell Signal **7**(6): 545-57.

Nakatani, K., D. A. Thompson, et al. (1999). "Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines." J Biol Chem **274**(31): 21528-32.

Natarajan, M., T. P. Hecker, et al. (2003). "FAK signaling in anaplastic astrocytoma and glioblastoma tumors." Cancer J **9**(2): 126-33.

Navin, N., J. Kendall, et al. (2011). "Tumour evolution inferred by single-cell sequencing." Nature **472**(7341): 90-4.

Nawijn, M. C., A. Alendar, et al. (2011). "For better or for worse: the role of Pim oncogenes in tumorigenesis." Nat Rev Cancer **11**(1): 23-34.

Negrini, S., V. G. Gorgoulis, et al. (2010). "Genomic instability--an evolving hallmark of cancer." Nat Rev Mol Cell Biol **11**(3): 220-8.

Nicklin, P., P. Bergman, et al. (2009). "Bidirectional transport of amino acids regulates mTOR and autophagy." Cell **136**(3): 521-34.

Niida, Y., A. O. Stemmer-Rachamimov, et al. (2001). "Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions." Am J Hum Genet **69**(3): 493-503.

Nishizuka, M., E. Arimoto, et al. (2003). "Crucial role of TCL/TC10beta L, a subfamily of Rho GTPase, in adipocyte differentiation." J Biol Chem **278**(17): 15279-84.

Nobukuni, T., S. C. Kozma, et al. (2007). "hvps34, an ancient player, enters a growing game: mTOR Complex1/S6K1 signaling." Curr Opin Cell Biol **19**(2): 135-41.

Notta, F., C. G. Mullighan, et al. (2011). "Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells." Nature **469**(7330): 362-7.

O'Brien, C. A., A. Kreso, et al. (2010). "Cancer stem cells and self-renewal." Clin Cancer Res **16**(12): 3113-20.

O'Reilly, T., P. M. McSheehy, et al. (2011). "Evaluation of the mTOR inhibitor, everolimus, in combination with cytotoxic antitumor agents using human tumor models in vitro and in vivo." Anticancer Drugs **22**(1): 58-78.

Oakhill, J. S., R. Steel, et al. (2011). "AMPK is a direct adenylate charge-regulated protein kinase." Science **332**(6036): 1433-5.

Oh, Y., S. R. Nagalla, et al. (1996). "Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II." J Biol Chem **271**(48): 30322-5.

Ohira, K., K. J. Homma, et al. (2006). "TrkB-T1 regulates the RhoA signaling and actin cytoskeleton in glioma cells." Biochem Biophys Res Commun **342**(3): 867-74.

Ohne, Y., T. Takahara, et al. (2008). "Isolation of hyperactive mutants of mammalian target of rapamycin." J Biol Chem **283**(46): 31861-70.

Ohtsuka, M., H. Fukumitsu, et al. (2009). "Neurotrophin-3 stimulates neurogenetic proliferation via the extracellular signal-regulated kinase pathway." J Neurosci Res **87**(2): 301-6.

Okamura, N., M. Hirai, et al. (1993). "Digoxin-cyclosporin A interaction: modulation of the multidrug transporter P-glycoprotein in the kidney." J Pharmacol Exp Ther **266**(3): 1614-9.

Okumura, E., T. Fukuhara, et al. (2002). "Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition." Nat Cell Biol **4**(2): 111-6.

Olsen, B. B., O. G. Issinger, et al. (2010). "Regulation of DNA-dependent protein kinase by protein kinase CK2 in human glioblastoma cells." Oncogene **29**(45): 6016-26.

Omholt, K., S. Karsberg, et al. (2002). "Screening of N-ras codon 61 mutations in paired primary and metastatic cutaneous melanomas: mutations occur early and persist throughout tumor progression." Clin Cancer Res **8**(11): 3468-74.

Onishi, H., T. Morisaki, et al. (2011). "Long-term vaccine therapy with autologous whole tumor cell-pulsed dendritic cells for a patient with recurrent rectal carcinoma." Anticancer Res **31**(11): 3995-4005.

Opel, D., M. A. Westhoff, et al. (2008). "Phosphatidylinositol 3-kinase inhibition broadly sensitizes glioblastoma cells to death receptor- and drug-induced apoptosis." Cancer Res **68**(15): 6271-80.

Orian-Rousseau, V. (2010). "CD44, a therapeutic target for metastasising tumours." Eur J Cancer **46**(7): 1271-7.

Osborne, C. K. (1998). "Tamoxifen in the treatment of breast cancer." N Engl J Med **339**(22): 1609-18.

Osisami, M., W. Ali, et al. (2012). "A role for phospholipase D3 in myotube formation." PLoS One **7**(3): e33341.

Otero, J. J. and T. Tihan (2011). "Morphological analysis of CDC2 and glycogen synthase kinase 3beta phosphorylation as markers of g2 --> m transition in glioma." Patholog Res Int **2011**: 216086.

Ozes, O. N., L. D. Mayo, et al. (1999). "NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase." Nature **401**(6748): 82-5.

Paez-Ribes, M., E. Allen, et al. (2009). "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis." Cancer Cell **15**(3): 220-31.

Pan, T., P. Rawal, et al. (2009). "Rapamycin protects against rotenone-induced apoptosis through autophagy induction." Neuroscience **164**(2): 541-51.

Paoloni, M. C., C. Mazcko, et al. (2010). "Rapamycin pharmacokinetic and pharmacodynamic relationships in osteosarcoma: a comparative oncology study in dogs." PLoS One **5**(6): e11013.

Papa, V., P. L. Tazzari, et al. (2008). "Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor perifosine in acute myelogenous leukemia cells." Leukemia **22**(1): 147-60.

Patard, J. J., F. Saint, et al. (1998). "Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review." Urol Res **26**(3): 155-9.

Patel, S., B. W. Doble, et al. (2008). "Tissue-specific role of glycogen synthase kinase 3beta in glucose homeostasis and insulin action." Mol Cell Biol **28**(20): 6314-28.

Patel, V., T. Lahusen, et al. (2002). "Perifosine, a novel alkylphospholipid, induces p21(WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest." Cancer Res **62**(5): 1401-9.

Patrawala, L., T. Calhoun, et al. (2005). "Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic." Cancer Res **65**(14): 6207-19.

Pearce, L. R., X. Huang, et al. (2007). "Identification of Protor as a novel Rictor-binding component of mTOR complex-2." Biochem J **405**(3): 513-22.

Pearce, L. R., D. Komander, et al. (2010). "The nuts and bolts of AGC protein kinases." Nat Rev Mol Cell Biol **11**(1): 9-22.

Pearce, L. R., E. M. Sommer, et al. (2011). "Protor-1 is required for efficient mTORC2-mediated activation of SGK1 in the kidney." Biochem J **436**(1): 169-79.

Pedersen, K. M., B. Finsen, et al. (1998). "Expression of a novel murine phospholipase D homolog coincides with late neuronal development in the forebrain." J Biol Chem **273**(47): 31494-504.

Pedrero, J. M., D. G. Carracedo, et al. (2005). "Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma." Int J Cancer **114**(2): 242-8.

Pelloski, C. E., E. Lin, et al. (2006). "Prognostic associations of activated mitogen-activated protein kinase and Akt pathways in glioblastoma." Clin Cancer Res **12**(13): 3935-41.

Pelosof, L. C. and D. E. Gerber (2010). "Paraneoplastic syndromes: an approach to diagnosis and treatment." Mayo Clin Proc **85**(9): 838-54.

Peltier, J., A. O'Neill, et al. (2007). "PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation." Dev Neurobiol **67**(10): 1348-61.

Pen, A., Y. Durocher, et al. (2011). "Insulin-like growth factor binding protein 7 exhibits tumor suppressive and vessel stabilization properties in U87MG and T98G glioblastoma cell lines." Cancer Biol Ther **12**(7): 634-46.

Pen, A., M. J. Moreno, et al. (2008). "Glioblastoma-secreted factors induce IGFBP7 and angiogenesis by modulating Smad-2-dependent TGF-beta signaling." Oncogene **27**(54): 6834-44.

Pen, A., M. J. Moreno, et al. (2007). "Molecular markers of extracellular matrix remodeling in glioblastoma vessels: microarray study of laser-captured glioblastoma vessels." Glia **55**(6): 559-72.

Perez, M. J., E. H. Ortiz, et al. (2009). "Fyn kinase is involved in oligodendroglial cell differentiation induced by apotransferrin." J Neurosci Res **87**(15): 3378-89.

Pfannschmidt, J., G. Egerer, et al. (2012). "Surgical intervention for pulmonary metastases." Dtsch Arztebl Int **109**(40): 645-51.

Philp, A. J., I. G. Campbell, et al. (2001). "The phosphatidylinositol 3'-kinase p85alpha gene is an oncogene in human ovarian and colon tumors." Cancer Res **61**(20): 7426-9.

Phoenix, T. N. and S. Temple (2010). "Spred1, a negative regulator of Ras-MAPK-ERK, is enriched in CNS germinal zones, dampens NSC proliferation, and maintains ventricular zone structure." Genes Dev **24**(1): 45-56.

Piccirillo, S. G., B. A. Reynolds, et al. (2006). "Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells." Nature **444**(7120): 761-5.

Pizzarello, D. J. and R. L. Witcofski (1972). Medical Radiation Biology, Philadelphia, Lea and Febiger.

Ploemacher, R. E., J. P. van der Sluijs, et al. (1989). "An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse." Blood **74**(8): 2755-63.

Poch, E., R. Minambres, et al. (2007). "RhoE interferes with Rb inactivation and regulates the proliferation and survival of the U87 human glioblastoma cell line." Exp Cell Res **313**(4): 719-31.

Poirier, M. B., G. Hamann, et al. (2005). "General receptor for phosphoinositides 1, a novel repressor of thyroid hormone receptor action that prevents deoxyribonucleic acid binding." Mol Endocrinol **19**(8): 1991-2005.

Pollak, M. (2012). "The insulin receptor/insulin-like growth factor receptor family as a therapeutic target in oncology." Clin Cancer Res **18**(1): 40-50.

Polyak, K. and W. C. Hahn (2006). "Roots and stems: stem cells in cancer." Nat Med **12**(3): 296-300.

Ponomaryov, T., A. Peled, et al. (2000). "Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function." J Clin Invest **106**(11): 1331-9.

Popovici, C., J. Adelaide, et al. (1998). "Fibroblast growth factor receptor 1 is fused to FIM in stem-cell myeloproliferative disorder with t(8;13)." Proc Natl Acad Sci U S A **95**(10): 5712-7.

Popovici, C., B. Zhang, et al. (1999). "The t(6;8)(q27;p11) translocation in a stem cell myeloproliferative disorder fuses a novel gene, FOP, to fibroblast growth factor receptor 1." Blood **93**(4): 1381-9.

Potapova, I. A., M. R. El-Maghrabi, et al. (2000). "Phosphorylation of recombinant human ATP:citrate lyase by cAMP-dependent protein kinase abolishes homotropic allosteric regulation of the enzyme by citrate and increases the enzyme activity. Allosteric activation of ATP:citrate lyase by phosphorylated sugars." Biochemistry **39**(5): 1169-79.

Powis, G., R. Bonjouklian, et al. (1994). "Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase." Cancer Res **54**(9): 2419-23.

Preiss, T. and M. W. Hentze (2003). "Starting the protein synthesis machine: eukaryotic translation initiation." Bioessays **25**(12): 1201-11.

Prigent, S. A., M. Nagane, et al. (1996). "Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway." J Biol Chem **271**(41): 25639-45.

Puc, J., M. Keniry, et al. (2005). "Lack of PTEN sequesters CHK1 and initiates genetic instability." Cancer Cell **7**(2): 193-204.

Pullen, N., P. B. Dennis, et al. (1998). "Phosphorylation and activation of p70s6k by PDK1." Science **279**(5351): 707-10.

Qi, X. J., G. M. Wildey, et al. (2006). "Evidence that Ser87 of BimEL is phosphorylated by Akt and regulates BimEL apoptotic function." J Biol Chem **281**(2): 813-23.

Qian, Z. Y., Y. Y. Wu, et al. (2008). "[Expression of SV40Tag, Rb and IRS-1 in glioma detected by tissue microarray and their relation with tumorigenesis and progression of gliomas]." Zhonghua Zhong Liu Za Zhi **30**(6): 432-6.

Radhakrishnan, Y., L. A. Maile, et al. (2008). "Insulin-like growth factor-I stimulates Shc-dependent phosphatidylinositol 3-kinase activation via Grb2-associated p85 in vascular smooth muscle cells." J Biol Chem **283**(24): 16320-31.

Rafalski, V. A. and A. Brunet (2011). "Energy metabolism in adult neural stem cell fate." Prog Neurobiol **93**(2): 182-203.

Rainov, N. G., S. Koch, et al. (2000). "Characterization of a canine glioma cell line as related to established experimental brain tumor models." J Neuropathol Exp Neurol **59**(7): 607-13.

Rajagopalan, P. T., Z. Zhang, et al. (2002). "Interaction of dihydrofolate reductase with methotrexate: ensemble and single-molecule kinetics." Proc Natl Acad Sci U S A **99**(21): 13481-6.

Rajan, P. and R. D. McKay (1998). "Multiple routes to astrocytic differentiation in the CNS." J Neurosci **18**(10): 3620-9.

Rajaram, S., D. J. Baylink, et al. (1997). "Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions." Endocr Rev **18**(6): 801-31.

Ram, R., G. Lorente, et al. (2006). "Discoidin domain receptor-1a (DDR1a) promotes glioma cell invasion and adhesion in association with matrix metalloproteinase-2." J Neurooncol **76**(3): 239-48.

Ramaswamy, S., N. Nakamura, et al. (2002). "A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR." Cancer Cell **2**(1): 81-91.

Ramirez-Valle, F., M. L. Badura, et al. (2010). "Mitotic raptor promotes mTORC1 activity, G(2)/M cell cycle progression, and internal ribosome entry site-mediated mRNA translation." Mol Cell Biol **30**(13): 3151-64.

Raught, B. and A. C. Gingras (1999). "eIF4E activity is regulated at multiple levels." Int J Biochem Cell Biol **31**(1): 43-57.

Raught, B., F. Peiretti, et al. (2004). "Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases." Embo J **23**(8): 1761-9.

Ravichandran, K. S. (2001). "Signaling via Shc family adapter proteins." Oncogene **20**(44): 6322-30.

Rebuzzi, L., M. Willmann, et al. (2007). "Detection of vascular endothelial growth factor (VEGF) and VEGF receptors Flt-1 and KDR in canine mastocytoma cells." Vet Immunol Immunopathol **115**(3-4): 320-33.

Reiling, J. H. and E. Hafen (2004). "The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila." Genes Dev **18**(23): 2879-92.

Rendtorff, N. D., B. Bjerregaard, et al. (2005). "Analysis of 65 tuberous sclerosis complex (TSC) patients by TSC2 DGGE, TSC1/TSC2 MLPA, and TSC1 long-range PCR sequencing, and report of 28 novel mutations." Hum Mutat **26**(4): 374-83.

Reusch, H. P., S. Zimmermann, et al. (2001). "Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells." J Biol Chem **276**(36): 33630-7.

Reya, T., S. J. Morrison, et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-11.

Reyland, M. E. (2007). "Protein kinase Cdelta and apoptosis." Biochem Soc Trans **35**(Pt 5): 1001-4.

Reynolds, B. A. and S. Weiss (1992). "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system." Science **255**(5052): 1707-10.

Rhodes, N., D. A. Heerding, et al. (2008). "Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity." Cancer Res **68**(7): 2366-74.

Richter, A., H. Murua Escobar, et al. (2005). "RAS gene hot-spot mutations in canine neoplasias." J Hered **96**(7): 764-5.

Rico-Bautista, E., A. Flores-Morales, et al. (2006). "Suppressor of cytokine signaling (SOCS) 2, a protein with multiple functions." Cytokine Growth Factor Rev **17**(6): 431-9.

Riento, K., P. Villalonga, et al. (2005). "Function and regulation of RhoE." Biochem Soc Trans **33**(Pt 4): 649-51.

Ries, S. J. and C. H. Brandts (2004). "Oncolytic viruses for the treatment of cancer: current strategies and clinical trials." Drug Discov Today **9**(17): 759-68.

Rizzieri, D. A., E. Feldman, et al. (2008). "A phase 2 clinical trial of deforolimus (AP23573, MK-8669), a novel mammalian target of rapamycin inhibitor, in patients with relapsed or refractory hematologic malignancies." Clin Cancer Res **14**(9): 2756-62.

Rodrigues, G. A., M. Falasca, et al. (2000). "A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling." Mol Cell Biol **20**(4): 1448-59.

Rodriguez-Viciana, P., P. H. Warne, et al. (1996). "Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation." Embo J **15**(10): 2442-51.

Rogers, G. W., Jr., A. A. Komar, et al. (2002). "eIF4A: the godfather of the DEAD box helicases." Prog Nucleic Acid Res Mol Biol **72**: 307-31.

Rokudai, S., N. Fujita, et al. (2002). "Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis." Mol Cell Biol **22**(24): 8695-708.

Rong, R., Q. He, et al. (2002). "TC21 mediates transformation and cell survival via activation of phosphatidylinositol 3-kinase/Akt and NF-kappaB signaling pathway." Oncogene **21**(7): 1062-70.

Roth, S. and R. Fodde (2011). "Quiescent stem cells in intestinal homeostasis and cancer." Cell Commun Adhes **18**(3): 33-44.

Rountree, C. B., W. Ding, et al. (2009). "Expansion of CD133-expressing liver cancer stem cells in liver-specific phosphatase and tensin homolog deleted on chromosome 10-deleted mice." Stem Cells **27**(2): 290-9.

Rouschop, K. M., L. Dubois, et al. (2011). "Deregulation of cap-dependent mRNA translation increases tumour radiosensitivity through reduction of the hypoxic fraction." Radiother Oncol **99**(3): 385-91.

Roux, P. P., B. A. Ballif, et al. (2004). "Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase." Proc Natl Acad Sci U S A **101**(37): 13489-94.

Roux, P. P. and J. Blenis (2004). "ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions." Microbiol Mol Biol Rev **68**(2): 320-44.

Roux, P. P., D. Shahbazian, et al. (2007). "RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation." J Biol Chem **282**(19): 14056-64.

Rozengurt, E. (2011). "Protein kinase D signaling: multiple biological functions in health and disease." Physiology (Bethesda) **26**(1): 23-33.

Rudd, J. A., J. Y. Tse, et al. (2000). "Cisplatin-induced emesis in the cat: effect of granisetron and dexamethasone." Eur J Pharmacol **391**(1-2): 145-50.

Rueda, D., B. Navarro, et al. (2002). "The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway." J Biol Chem **277**(48): 46645-50.

Sachs, A. B. and G. Varani (2000). "Eukaryotic translation initiation: there are (at least) two sides to every story." Nat Struct Biol **7**(5): 356-61.

Sai, K., S. Wang, et al. (2012). "Induction of cell-cycle arrest and apoptosis in glioblastoma stem-like cells by WP1193, a novel small molecule inhibitor of the JAK2/STAT3 pathway." J Neurooncol **107**(3): 487-501.

Saif, M. W. (2011). "Pancreatic neoplasm in 2011: an update." JOP **12**(4): 316-21.

Saito, Y. D., A. R. Jensen, et al. (2010). "Fyn: a novel molecular target in cancer." Cancer **116**(7): 1629-37.

Sakai, A., C. Thieblemont, et al. (1998). "PTEN gene alterations in lymphoid neoplasms." Blood **92**(9): 3410-5.

Salazar, M., A. Carracedo, et al. (2009). "Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells." J Clin Invest **119**(5): 1359-72.

Saletta, F., Y. Suryo Rahmanto, et al. (2010). "The translational regulator eIF3a: the tricky eIF3 subunit!" Biochim Biophys Acta **1806**(2): 275-86.

Salhia, B., F. Rutten, et al. (2005). "Inhibition of Rho-kinase affects astrocytoma morphology, motility, and invasion through activation of Rac1." Cancer Res **65**(19): 8792-800.

Sampaio, C., M. Dance, et al. (2008). "Signal strength dictates phosphoinositide 3-kinase contribution to Ras/extracellular signal-regulated kinase 1 and 2 activation

via differential Gab1/Shp2 recruitment: consequences for resistance to epidermal growth factor receptor inhibition." Mol Cell Biol **28**(2): 587-600.

Samuels, I. S., J. C. Karlo, et al. (2008). "Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function." J Neurosci **28**(27): 6983-95.

Samuels, Y., Z. Wang, et al. (2004). "High frequency of mutations of the PIK3CA gene in human cancers." Science **304**(5670): 554.

Sancak, Y., T. R. Peterson, et al. (2008). "The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1." Science **320**(5882): 1496-501.

Sancak, Y., C. C. Thoreen, et al. (2007). "PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase." Mol Cell **25**(6): 903-15.

Sanchez-Margalet, V., I. D. Goldfine, et al. (1994). "Role of phosphatidylinositol-3-kinase in insulin receptor signaling: studies with inhibitor, LY294002." Biochem Biophys Res Commun **204**(2): 446-52.

Sarbassov, D. D., S. M. Ali, et al. (2006). "Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB." Mol Cell **22**(2): 159-68.

Sarbassov, D. D., D. A. Guertin, et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-101.

Sarkar, S. and V. W. Yong (2010). "Reduction of protein kinase C delta attenuates tenascin-C stimulated glioma invasion in three-dimensional matrix." Carcinogenesis **31**(2): 311-7.

Sasayama, T., M. Nishihara, et al. (2009). "MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC." Int J Cancer **125**(6): 1407-13.

Sato, S., N. Fujita, et al. (2000). "Modulation of Akt kinase activity by binding to Hsp90." Proc Natl Acad Sci U S A **97**(20): 10832-7.

Sausville, E. A. and D. L. Longo (2001). Principles of cancer treatment. Harrison's principles of internal medicine. E. Braunwald, A. S. Fauci, D. L. Kasperet al. U.S.A, The McGraw-Hill Companies, Inc. **1:** 530-546.

Sauvageot, C. M., J. L. Weatherbee, et al. (2009). "Efficacy of the HSP90 inhibitor 17-AAG in human glioma cell lines and tumorigenic glioma stem cells." Neuro Oncol **11**(2): 109-21.

Schadendorf, D., S. M. Algarra, et al. (2009). "Immunotherapy of distant metastatic disease." Ann Oncol **20 Suppl 6**: vi41-50.

Schlenska-Lange, A., H. Knupfer, et al. (2008). "Cell proliferation and migration in glioblastoma multiforme cell lines are influenced by insulin-like growth factor I in vitro." Anticancer Res **28**(2A): 1055-60.

Schlessinger, J. (2000). "Cell signaling by receptor tyrosine kinases." Cell **103**(2): 211-25.

Schmidt, M., S. Fernandez de Mattos, et al. (2002). "Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D." Mol Cell Biol **22**(22): 7842-52.

Schmidt, S., J. Rainer, et al. (2004). "Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance." Cell Death Differ **11 Suppl 1**: S45-55.

Schneider, U., H. U. Schwenk, et al. (1977). "Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma." Int J Cancer **19**(5): 621-6.

Schultz, R. M., R. L. Merriman, et al. (1995). "In vitro and in vivo antitumor activity of the phosphatidylinositol-3-kinase inhibitor, wortmannin." Anticancer Res **15**(4): 1135-9.

Schvartzman, J. M., R. Sotillo, et al. (2010). "Mitotic chromosomal instability and cancer: mouse modelling of the human disease." Nat Rev Cancer **10**(2): 102-15.

Sciaccaluga, M., G. L. Gianfranceschi, et al. (2007). "Constitutive phosphorylation of Janus kinase 2 in the GL15 glioblastoma derived human cell line." Oncol Rep **17**(1): 17-23.

Scott, P. H., G. J. Brunn, et al. (1998). "Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway." Proc Natl Acad Sci U S A **95**(13): 7772-7.

Seigel, G. M., L. M. Campbell, et al. (2005). "Cancer stem cell characteristics in retinoblastoma." Mol Vis **11**: 729-37.

Seki, Y., K. Hayashi, et al. (2002). "Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation." Proc Natl Acad Sci U S A **99**(20): 13003-8.

Semenza, G. L. (2003). "Targeting HIF-1 for cancer therapy." Nat Rev Cancer **3**(10): 721-32.

Senft, C., M. Priester, et al. (2011). "Inhibition of the JAK-2/STAT3 signaling pathway impedes the migratory and invasive potential of human glioblastoma cells." J Neurooncol **101**(3): 393-403.

Shah, O. J., Z. Wang, et al. (2004). "Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies." Curr Biol **14**(18): 1650-6.

Shan, X., M. J. Czar, et al. (2000). "Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation." Mol Cell Biol **20**(18): 6945-57.

Shao, Y. and A. E. Aplin (2010). "Akt3-mediated resistance to apoptosis in B-RAF-targeted melanoma cells." Cancer Res **70**(16): 6670-81.

Shaw, D. R. and T. V. Strong (2006). "DNA vaccines for cancer." Front Biosci **11**: 1189-98.

Shay, J. W., Y. Zou, et al. (2001). "Telomerase and cancer." Hum Mol Genet **10**(7): 677-85.

Shen, Q., S. K. Goderie, et al. (2004). "Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells." Science **304**(5675): 1338-40.

Sheng, Z., L. Li, et al. (2010). "A genome-wide RNA interference screen reveals an essential CREB3L2-ATF5-MCL1 survival pathway in malignant glioma with therapeutic implications." Nat Med **16**(6): 671-7.

Sherbakova, E. A., T. P. Stromskaia, et al. (2008). "[Role of PTEN protein in multidrug resistance of prostate cancer cells]." Mol Biol (Mosk) **42**(3): 487-93.

Sheridan, C. (2011). "Gene therapy finds its niche." Nat Biotechnol **29**(2): 121-8.

Sherry, M. M., A. Reeves, et al. (2009). "STAT3 is required for proliferation and maintenance of multipotency in glioblastoma stem cells." Stem Cells **27**(10): 2383-92.

Shi, Z., X. Qian, et al. (2012). "Nuclear Translocation of beta-catenin is Essential for Glioma Cell Survival." J Neuroimmune Pharmacol.

Shimada, S., H. Yamamura, et al. (2004). "[Functional analysis of acid sensing ion channels]." Nihon Shinkei Seishin Yakurigaku Zasshi **24**(4): 243-6.

Shimano, K., M. Satake, et al. (2003). "Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1." Am J Pathol **163**(1): 3-9.

Shintani, T. and D. J. Klionsky (2004). "Autophagy in health and disease: a double-edged sword." Science **306**(5698): 990-5.

Shor, B., W. G. Zhang, et al. (2008). "A new pharmacologic action of CCI-779 involves FKBP12-independent inhibition of mTOR kinase activity and profound repression of global protein synthesis." Cancer Res **68**(8): 2934-43.

Sieber, C., J. Kopf, et al. (2009). "Recent advances in BMP receptor signaling." Cytokine Growth Factor Rev **20**(5-6): 343-55.

Silva, A., J. A. Yunes, et al. (2008). "PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability." J Clin Invest **118**(11): 3762-74.

Simmons, D. L., A. B. Satterthwaite, et al. (1992). "Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells." J Immunol **148**(1): 267-71.

Simpson, L. and R. Parsons (2001). "PTEN: life as a tumor suppressor." Exp Cell Res **264**(1): 29-41.

Singh, S. K., I. D. Clarke, et al. (2003). "Identification of a cancer stem cell in human brain tumors." Cancer Res **63**(18): 5821-8.

Skommer, J., D. Wlodkowic, et al. (2007). "Larger than life: Mitochondria and the Bcl-2 family." Leuk Res **31**(3): 277-86.

Skorski, T., A. Bellacosa, et al. (1997). "Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway." Embo J **16**(20): 6151-61.

Sofer, A., K. Lei, et al. (2005). "Regulation of mTOR and cell growth in response to energy stress by REDD1." Mol Cell Biol **25**(14): 5834-45.

Sondak, V. K. and J. A. Sosman (2003). "Results of clinical trials with an allogenic melanoma tumor cell lysate vaccine: Melacine." Semin Cancer Biol **13**(6): 409-15.

Song, H. and A. Moon (2006). "Glial cell-derived neurotrophic factor (GDNF) promotes low-grade Hs683 glioma cell migration through JNK, ERK-1/2 and p38 MAPK signaling pathways." Neurosci Res **56**(1): 29-38.

Sorrells, D. L., C. Meschonat, et al. (1999). "Pattern of amplification and overexpression of the eukaryotic initiation factor 4E gene in solid tumor." J Surg Res **85**(1): 37-42.

Sporn, M. B. and A. B. Roberts (1985). "Autocrine growth factors and cancer." Nature **313**(6005): 745-7.

Stanton, B. Z. and L. F. Peng (2010). "Small-molecule modulators of the Sonic Hedgehog signaling pathway." Mol Biosyst **6**(1): 44-54.

Stein, L., K. Roy, et al. (2011). "Clinical gene therapy for the treatment of RPE65-associated Leber congenital amaurosis." Expert Opin Biol Ther **11**(3): 429-39.

Stein, R. C. (2001). "Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment." Endocr Relat Cancer **8**(3): 237-48.

Stetler-Stevenson, W. G. (1999). "Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention." J Clin Invest **103**(9): 1237-41.

Stoica, G., J. Levine, et al. (2011). "Canine astrocytic tumors: a comparative review." Vet Pathol **48**(1): 266-75.

Stoler, D. L., N. Chen, et al. (1999). "The onset and extent of genomic instability in sporadic colorectal tumor progression." Proc Natl Acad Sci U S A **96**(26): 15121-6.

Storms, R. W., M. A. Goodell, et al. (2000). "Hoechst dye efflux reveals a novel CD7(+)CD34(-) lymphoid progenitor in human umbilical cord blood." Blood **96**(6): 2125-33.

Strandstrom, H. V. and E. Rimaila-Parnanen (1979). "Canine atypical malignant lymphoma." Am J Vet Res **40**(7): 1033-4.

Strik, H. M., M. Weller, et al. (2000). "Heat shock protein expression in human gliomas." Anticancer Res **20**(6B): 4457-62.

Strong, J. A. (1963). "HORMONAL CONTROL OF CANCER." Proc R Soc Med **56**: 665-70.

Stupp, R., W. P. Mason, et al. (2005). "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma." N Engl J Med **352**(10): 987-96.

Sturla, L. M., P. O. Zinn, et al. (2011). "Src homology domain-containing phosphatase 2 suppresses cellular senescence in glioblastoma." Br J Cancer **105**(8): 1235-43.

Sugawa, N., A. J. Ekstrand, et al. (1990). "Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas." Proc Natl Acad Sci U S A **87**(21): 8602-6.

Suire, S., J. Coadwell, et al. (2005). "p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma." Curr Biol **15**(6): 566-70.

Sumi, T., Y. Fujimoto, et al. (2004). "STAT3 is dispensable for maintenance of self-renewal in nonhuman primate embryonic stem cells." Stem Cells **22**(5): 861-72.

Summer, R., D. N. Kotton, et al. (2003). "Side population cells and Bcrp1 expression in lung." Am J Physiol Lung Cell Mol Physiol **285**(1): L97-104.

Sun, M., G. Wang, et al. (2001). "AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells." Am J Pathol **159**(2): 431-7.

Sun, S. Y., L. M. Rosenberg, et al. (2005). "Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition." Cancer Res **65**(16): 7052-8.

Sundqvist, A., M. T. Bengoechea-Alonso, et al. (2005). "Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7)." Cell Metab **1**(6): 379-91.

Swanson, K. D., Y. Tang, et al. (2008). "The Skap-hom dimerization and PH domains comprise a 3'-phosphoinositide-gated molecular switch." Mol Cell **32**(4): 564-75.

Tabatabai, G., M. Weller, et al. (2010). "Targeting integrins in malignant glioma." Target Oncol **5**(3): 175-81.

Taddei, M. L., E. Giannoni, et al. (2013). "Microenvironment and tumor cell plasticity: An easy way out." Cancer Lett.

Taha, C., Z. Liu, et al. (1999). "Opposite translational control of GLUT1 and GLUT4 glucose transporter mRNAs in response to insulin. Role of mammalian target of rapamycin, protein kinase b, and phosphatidylinositol 3-kinase in GLUT1 mRNA translation." J Biol Chem **274**(46): 33085-91.

Taichman, R. S., C. Cooper, et al. (2002). "Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone." Cancer Res **62**(6): 1832-7.

Takahashi, S. (2011). "Vascular endothelial growth factor (VEGF), VEGF receptors and their inhibitors for antiangiogenic tumor therapy." Biol Pharm Bull **34**(12): 1785-8.

Takesono, A., L. D. Finkelstein, et al. (2002). "Beyond calcium: new signaling pathways for Tec family kinases." J Cell Sci **115**(Pt 15): 3039-48.

Takeuchi, H., Y. Kondo, et al. (2005). "Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors." Cancer Res **65**(8): 3336-46.

Tamburini, J., N. Chapuis, et al. (2008). "Mammalian target of rapamycin (mTOR) inhibition activates phosphatidylinositol 3-kinase/Akt by up-regulating insulin-like growth factor-1 receptor signaling in acute myeloid leukemia: rationale for therapeutic inhibition of both pathways." Blood **111**(1): 379-82.

Tang, D. G. (2012). "Understanding cancer stem cell heterogeneity and plasticity." Cell Res **22**(3): 457-472.

Tang, J., P. Bouyer, et al. (2010). "Activated PKC{delta} and PKC{epsilon} inhibit epithelial chloride secretion response to cAMP via inducing internalization of the Na+-K+-2Cl- cotransporter NKCC1." J Biol Chem **285**(44): 34072-85.

Tang, S. N., J. Fu, et al. (2012). "Inhibition of sonic hedgehog pathway and pluripotency maintaining factors regulate human pancreatic cancer stem cell characteristics." Int J Cancer.

Tang, T. T., D. Dowbenko, et al. (2002). "The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor." J Biol Chem **277**(16): 14255-65.

Taussig, D. C., D. J. Pearce, et al. (2005). "Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia." Blood **106**(13): 4086-92.

Taylor, V., M. Wong, et al. (2000). "5' phospholipid phosphatase SHIP-2 causes protein kinase B inactivation and cell cycle arrest in glioblastoma cells." Mol Cell Biol **20**(18): 6860-71.

Terada, N., H. R. Patel, et al. (1994). "Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins." Proc Natl Acad Sci U S A **91**(24): 11477-81.

Terunuma, A., K. L. Jackson, et al. (2003). "Side population keratinocytes resembling bone marrow side population stem cells are distinct from label-retaining keratinocyte stem cells." J Invest Dermatol **121**(5): 1095-103.

Tervonen, T. A., F. Ajamian, et al. (2006). "Overexpression of a truncated TrkB isoform increases the proliferation of neural progenitors." Eur J Neurosci **24**(5): 1277-85.

Theon, A. (2000). Pratical radiation therapy. Textbook of veterinary internal medicine: diseases of the dog and cat. S. J. Ettinger and E. C. Feldman. Philadelphia, Pennsylvania, USA, W.B. Saunders Company. **1:** 489-98.

Tolias, K. F., L. C. Cantley, et al. (1995). "Rho family GTPases bind to phosphoinositide kinases." J Biol Chem **270**(30): 17656-9.

Toyama, S., N. Tamura, et al. (2010). "Inhibitory effects of ZSTK474, a novel phosphoinositide 3-kinase inhibitor, on osteoclasts and collagen-induced arthritis in mice." Arthritis Res Ther **12**(3): R92.

Tremblay, F., S. Brule, et al. (2007). "Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance." Proc Natl Acad Sci U S A **104**(35): 14056-61.

Triel, C., M. E. Vestergaard, et al. (2004). "Side population cells in human and mouse epidermis lack stem cell characteristics." Exp Cell Res **295**(1): 79-90.

Trisciuoglio, D., B. Uranchimeg, et al. (2008). "Induction of apoptosis in human cancer cells by candidaspongiolide, a novel sponge polyketide." J Natl Cancer Inst **100**(17): 1233-46.

Tsang, C. K., H. Qi, et al. (2007). "Targeting mammalian target of rapamycin (mTOR) for health and diseases." Drug Discov Today **12**(3-4): 112-24.

Tsonis, P. A. (2007). "Bridging knowledge gaps on the long road to regeneration: classical models meet stem cell manipulation and bioengineering." Mol Interv **7**(5): 249-50.

Tsuruta, F., N. Masuyama, et al. (2002). "The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria." J Biol Chem **277**(16): 14040-7.

Tybulewicz, V. L. (2005). "Vav-family proteins in T-cell signalling." Curr Opin Immunol **17**(3): 267-74.

Ueda, T., R. Watanabe-Fukunaga, et al. (2004). "Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development." Mol Cell Biol **24**(15): 6539-49.

Uyeno, S., J. Komura, et al. (1996). "Alteration of c-fos gene methylation in human gliomas." Mol Carcinog **16**(2): 91-100.

van der Vos, K. E. and P. J. Coffer (2011). "The extending network of FOXO transcriptional target genes." Antioxid Redox Signal **14**(4): 579-92.

Vander Haar, E., S. I. Lee, et al. (2007). "Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40." Nat Cell Biol **9**(3): 316-23.

VanderKuur, J., G. Allevato, et al. (1995). "Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb2." J Biol Chem **270**(13): 7587-93.

Vanhaesebroeck, B., J. Guillermet-Guibert, et al. (2010). "The emerging mechanisms of isoform-specific PI3K signalling." Nat Rev Mol Cell Biol **11**(5): 329-41.

Vasiliou, V., K. Vasiliou, et al. (2009). "Human ATP-binding cassette (ABC) transporter family." Hum Genomics **3**(3): 281-90.

Vazquez, F., S. R. Grossman, et al. (2001). "Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex." J Biol Chem **276**(52): 48627-30.

Veilleux, A., V. P. Houde, et al. (2010). "Chronic inhibition of the mTORC1/S6K1 pathway increases insulin-induced PI3K activity but inhibits Akt2 and glucose transport stimulation in 3T3-L1 adipocytes." Mol Endocrinol **24**(4): 766-78.

Vermeulen, L., E. M. F. De Sousa, et al. (2010). "Wnt activity defines colon cancer stem cells and is regulated by the microenvironment." Nat Cell Biol **12**(5): 468-76.

Vichalkovski, A., E. Gresko, et al. (2010). "PKB/AKT phosphorylation of the transcription factor Twist-1 at Ser42 inhibits p53 activity in response to DNA damage." Oncogene **29**(24): 3554-65.

Vogel, U. S., R. A. Dixon, et al. (1988). "Cloning of bovine GAP and its interaction with oncogenic ras p21." Nature **335**(6185): 90-3.

Voigt, P., C. Brock, et al. (2005). "Assigning functional domains within the p101 regulatory subunit of phosphoinositide 3-kinase gamma." J Biol Chem **280**(6): 5121-7.

Wagers, A. J. and I. L. Weissman (2004). "Plasticity of adult stem cells." Cell **116**(5): 639-48.

Wallin, J. J., J. Guan, et al. (2010). "Nuclear phospho-Akt increase predicts synergy of PI3K inhibition and doxorubicin in breast and ovarian cancer." Sci Transl Med **2**(48): 48ra66.

Wang, B., Y. Gao, et al. (2009). "Erk1/2 promotes proliferation and inhibits neuronal differentiation of neural stem cells." Neurosci Lett **461**(3): 252-7.

Wang, G. K., L. Hu, et al. (2006). "An interaction between insulin-like growth factor-binding protein 2 (IGFBP2) and integrin alpha5 is essential for IGFBP2-induced cell mobility." J Biol Chem **281**(20): 14085-91.

Wang, H., W. Shen, et al. (2003). "Insulin-like growth factor binding protein 2 enhances glioblastoma invasion by activating invasion-enhancing genes." Cancer Res **63**(15): 4315-21.

Wang, J. M., J. R. Chao, et al. (1999). "The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB." Mol Cell Biol **19**(9): 6195-206.

Wang, J. M., Y. S. Zeng, et al. (2007). "Recombinant adenovirus vector-mediated functional expression of neurotropin-3 receptor (TrkC) in neural stem cells." Exp Neurol **203**(1): 123-7.

Wang, X., L. E. Campbell, et al. (1998). "Amino acid availability regulates p70 S6 kinase and multiple translation factors." Biochem J **334 (Pt 1)**: 261-7.

Wang, X., W. Li, et al. (2001). "Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase." Embo J **20**(16): 4370-9.

Wang, X., P. Yue, et al. (2007). "Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation." Mol Cell Biol **27**(21): 7405-13.

Wang, Y. (2010). "Everolimus in renal cell carcinoma." Drugs Today (Barc) **46**(8): 557-66.

Wang, Y., C. Hagel, et al. (1998). "Trk A, B, and C are commonly expressed in human astrocytes and astrocytic gliomas but not by human oligodendrocytes and oligodendroglioma." Acta Neuropathol **96**(4): 357-64.

Wang, Y. A., S. K. Johnson, et al. (2009). "Differential enhancement of the anti-cancer effect of doxorubicin by Akt inhibitors on human breast cancer cells with differing genetic backgrounds." Oncol Rep **21**(2): 437-42.

Warne, P. H., P. R. Viciana, et al. (1993). "Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro." Nature **364**(6435): 352-5.

Waskiewicz, A. J., A. Flynn, et al. (1997). "Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2." Embo J **16**(8): 1909-20.

Weiler, M., O. Bahr, et al. (2006). "BCL-xL: time-dependent dissociation between modulation of apoptosis and invasiveness in human malignant glioma cells." Cell Death Differ **13**(7): 1156-69.

Weiner, H. L., H. Huang, et al. (2000). "Consistent and selective expression of the discoidin domain receptor-1 tyrosine kinase in human brain tumors." Neurosurgery **47**(6): 1400-9.

Weinstein, I. B. (2002). "Cancer. Addiction to oncogenes--the Achilles heal of cancer." Science **297**(5578): 63-4.

Wen, P. Y. and S. Kesari (2008). "Malignant gliomas in adults." N Engl J Med **359**(5): 492-507.

Wennerberg, K. and C. J. Der (2004). "Rho-family GTPases: it's not only Rac and Rho (and I like it)." J Cell Sci **117**(Pt 8): 1301-12.

Wesche, J., K. Haglund, et al. (2011). "Fibroblast growth factors and their receptors in cancer." Biochem J **437**(2): 199-213.

Westphal, M. and K. Lamszus (2011). "The neurobiology of gliomas: from cell biology to the development of therapeutic approaches." Nat Rev Neurosci **12**(9): 495-508.

Wheeler, A. P. and A. J. Ridley (2004). "Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility." Exp Cell Res **301**(1): 43-9.

Whiteman, E. L., H. Cho, et al. (2002). "Role of Akt/protein kinase B in metabolism." Trends Endocrinol Metab **13**(10): 444-51.

Wick, W., U. Naumann, et al. (2006). "Transforming growth factor-beta: a molecular target for the future therapy of glioblastoma." Curr Pharm Des **12**(3): 341-9.

Widmann, C., S. Gibson, et al. (1998). "Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals." J Biol Chem **273**(12): 7141-7.

Wilker, E. and M. B. Yaffe (2004). "14-3-3 Proteins--a focus on cancer and human disease." J Mol Cell Cardiol **37**(3): 633-42.

Withrow, S. J. and D. M. Vail, Eds. (2007). Withrow and MacEwen's Small Animal Clinical Oncology St. Louis, Missouri, Saunders Elsevier.

Witzig, T. E., S. M. Geyer, et al. (2005). "Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma." J Clin Oncol **23**(23): 5347-56.

Wolff, N. C., S. Vega-Rubin-de-Celis, et al. (2011). "Cell-type-dependent regulation of mTORC1 by REDD1 and the tumor suppressors TSC1/TSC2 and LKB1 in response to hypoxia." Mol Cell Biol **31**(9): 1870-84.

Wong, A. J., J. M. Ruppert, et al. (1992). "Structural alterations of the epidermal growth factor receptor gene in human gliomas." Proc Natl Acad Sci U S A **89**(7): 2965-9.

Woodcock, S. A. and D. A. Hughes (2004). "p120 Ras GTPase-activating protein associates with fibroblast growth factor receptors in Drosophila." Biochem J **380**(Pt 3): 767-74.

Wotring, L. L., L. B. Townsend, et al. (1990). "Dual mechanisms of inhibition of DNA synthesis by triciribine." Cancer Res **50**(16): 4891-9.

Wu, C. J., Z. Chen, et al. (2000). "Inhibition of EGFR-mediated phosphoinositide-3-OH kinase (PI3-K) signaling and glioblastoma phenotype by signal-regulatory proteins (SIRPs)." Oncogene **19**(35): 3999-4010.

Wu, C. J., D. M. O'Rourke, et al. (2001). "The tyrosine phosphatase SHP-2 is required for mediating phosphatidylinositol 3-kinase/Akt activation by growth factors." Oncogene **20**(42): 6018-25.

Wymann, M. P., G. Bulgarelli-Leva, et al. (1996). "Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction." Mol Cell Biol **16**(4): 1722-33.

Wymann, M. P. and L. Pirola (1998). "Structure and function of phosphoinositide 3-kinases." Biochim Biophys Acta **1436**(1-2): 127-50.

Xiang, F., J. Tanaka, et al. (2001). "Expression of vascular endothelial growth factor (VEGF) and its two receptors in diffusely infiltrating astrocytomas and relationship to proliferative activity of tumor cells." Brain Tumor Pathol **18**(2): 67-71.

Xie, D., D. Yin, et al. (2004). "Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and beta-catenin-TCF/Lef signaling pathways." Cancer Res **64**(6): 1987-96.

Xie, D., D. Yin, et al. (2004). "Levels of expression of CYR61 and CTGF are prognostic for tumor progression and survival of individuals with gliomas." Clin Cancer Res **10**(6): 2072-81.

Xu, B., A. Bhattacharjee, et al. (2003). "Interleukin-13 induction of 15-lipoxygenase gene expression requires p38 mitogen-activated protein kinase-mediated serine 727 phosphorylation of Stat1 and Stat3." Mol Cell Biol **23**(11): 3918-28.

Xu, C. X., L. Zhao, et al. (2011). "Augmentation of NVP-BEZ235's anticancer activity against human lung cancer cells by blockage of autophagy." Cancer Biol Ther **12**(6): 549-55.

Xu, L., D. Salloum, et al. (2011). "Phospholipase D Mediates Nutrient Input to Mammalian Target of Rapamycin Complex 1 (mTORC1)." J Biol Chem **286**(29): 25477-86.

Xu, Y., K. He, et al. (2011). "Telomerase targeted therapy in cancer and cancer stem cells." Clin Adv Hematol Oncol **9**(6): 442-55.

Xue, Y., N. L. Li, et al. (2011). "Phosphatidylinositol 3'-kinase signaling pathway is essential for Rac1-induced hypoxia-inducible factor-1(alpha) and vascular endothelial growth factor expression." Am J Physiol Heart Circ Physiol **300**(6): H2169-76.

Yadav, V. and M. F. Denning (2011). "Fyn is induced by Ras/PI3K/Akt signaling and is required for enhanced invasion/migration." Mol Carcinog **50**(5): 346-52.

Yaguchi, S., Y. Fukui, et al. (2006). "Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor." J Natl Cancer Inst **98**(8): 545-56.

Yamada, E., S. Okada, et al. (2005). "Akt2 phosphorylates Synip to regulate docking and fusion of GLUT4-containing vesicles." J Cell Biol **168**(6): 921-8.

Yamada, N., M. Kato, et al. (1996). "Bone morphogenetic protein type IB receptor is progressively expressed in malignant glioma tumours." Br J Cancer **73**(5): 624-9.

Yamada, S. M., F. Yamaguchi, et al. (1999). "Suppression of glioblastoma cell growth following antisense oligonucleotide-mediated inhibition of fibroblast growth factor receptor expression." Glia **28**(1): 66-76.

Yamada, S. M., F. Yamaguchi, et al. (1998). "[Inhibition of fibroblast growth factor receptor 1 expression in human glioblastoma cell contributes to the cell growth suppression]." No To Shinkei **50**(12): 1101-5.

Yan, B., H. H. Chour, et al. (2006). "RhoA protein expression correlates positively with degree of malignancy in astrocytomas." Neurosci Lett **407**(2): 124-6.

Yan, B., M. Zemskova, et al. (2003). "The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death." J Biol Chem **278**(46): 45358-67.

Yan, J., H. Kuroyanagi, et al. (1999). "Mouse ULK2, a novel member of the UNC-51-like protein kinases: unique features of functional domains." Oncogene **18**(43): 5850-9.

Yan, Z., S. A. Fedorov, et al. (2000). "PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with Cdc6 and modulates DNA replication in human cells." Mol Cell Biol **20**(3): 1021-9.

Yang, L., H. C. Dan, et al. (2004). "Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt." Cancer Res **64**(13): 4394-9.

Yang, Q., K. Inoki, et al. (2006). "Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity." Genes Dev **20**(20): 2820-32.

Yang, X., W. Cao, et al. (2009). "Isoform-specific expression of 14-3-3 proteins in human astrocytoma." J Neurol Sci **276**(1-2): 54-9.

Yang, Y., K. Iwanaga, et al. (2008). "Phosphatidylinositol 3-kinase mediates bronchioalveolar stem cell expansion in mouse models of oncogenic K-ras-induced lung cancer." PLoS One **3**(5): e2220.

Yao, J. C., M. H. Shah, et al. (2011). "Everolimus for advanced pancreatic neuroendocrine tumors." N Engl J Med **364**(6): 514-23.

Yao, Y. Q., X. Ding, et al. (2008). "Anti-tumor effect of beta-elemene in glioblastoma cells depends on p38 MAPK activation." Cancer Lett **264**(1): 127-34.

Yao, Y. Q., Y. H. Xu, et al. (2008). "[Effect of p38 MAPK on elemene-induced cell cycle arrest in C6 glioblastoma cells]." Zhonghua Yi Xue Za Zhi **88**(1): 56-8.

Yilmaz, O. H., R. Valdez, et al. (2006). "Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells." Nature **441**(7092): 475-82.

Yoeli-Lerner, M., G. K. Yiu, et al. (2005). "Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT." Mol Cell **20**(4): 539-50.

Yokogami, K., S. Wakisaka, et al. (2000). "Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR." Curr Biol **10**(1): 47-50.

Yoo, M. H. and D. L. Hatfield (2008). "The cancer stem cell theory: is it correct?" Mol Cells **26**(5): 514-6.

Yoon, C. H., K. H. Hyun, et al. (2011). "The small GTPase Rac1 is involved in the maintenance of stemness and malignancies in glioma stem-like cells." FEBS Lett **585**(14): 2331-8.

Yoshikawa, Y., T. Satoh, et al. (2007). "The M-Ras-RA-GEF-2-Rap1 pathway mediates tumor necrosis factor-alpha dependent regulation of integrin activation in splenocytes." Mol Biol Cell **18**(8): 2949-59.

Yoshino, Y., M. Aoyagi, et al. (2006). "Activation of p38 MAPK and/or JNK contributes to increased levels of VEGF secretion in human malignant glioma cells." Int J Oncol **29**(4): 981-7.

You, H., M. Pellegrini, et al. (2006). "FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal." J Exp Med **203**(7): 1657-63.

Young, N., D. K. Pearl, et al. (2009). "Sphingosine-1-phosphate regulates glioblastoma cell invasiveness through the urokinase plasminogen activator system and CCN1/Cyr61." Mol Cancer Res **7**(1): 23-32.

Young, N. and J. R. Van Brocklyn (2007). "Roles of sphingosine-1-phosphate (S1P) receptors in malignant behavior of glioma cells. Differential effects of S1P2 on cell migration and invasiveness." Exp Cell Res **313**(8): 1615-27.

Yu, S., P. Pu, et al. (1999). "[Observations on expression of c-fos and c-myc genes and activity of PDGFBB autocrine loop in 67 human gliomas]." Zhonghua Bing Li Xue Za Zhi **28**(3): 182-6.

Yuan, R., A. Kay, et al. (2009). "Targeting tumorigenesis: development and use of mTOR inhibitors in cancer therapy." J Hematol Oncol **2**: 45.

Yue, P. and J. Turkson (2009). "Targeting STAT3 in cancer: how successful are we?" Expert Opin Investig Drugs **18**(1): 45-56.

Zadjali, F., A. C. Pike, et al. (2011). "Structural basis for c-KIT inhibition by the suppressor of cytokine signaling 6 (SOCS6) ubiquitin ligase." J Biol Chem **286**(1): 480-90.

Zelzer, E., Y. Levy, et al. (1998). "Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT." Embo J **17**(17): 5085-94.

Zeng, Z., I. J. Samudio, et al. (2006). "Simultaneous inhibition of PDK1/AKT and Fms-like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and apoptosis in acute myelogenous leukemia." Cancer Res **66**(7): 3737-46.

Zhan, Y., G. J. Counelis, et al. (2009). "The protein tyrosine phosphatase SHP-2 is required for EGFRvIII oncogenic transformation in human glioblastoma cells." Exp Cell Res **315**(14): 2343-57.

Zhan, Y. and D. M. O'Rourke (2004). "SHP-2-dependent mitogen-activated protein kinase activation regulates EGFRvIII but not wild-type epidermal growth factor receptor phosphorylation and glioblastoma cell survival." Cancer Res **64**(22): 8292-8.

Zhang, H., G. Cicchetti, et al. (2003). "Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR." J Clin Invest **112**(8): 1223-33.

Zhang, J., S. Chen, et al. (2009). "[Over-expression of phospholipase D3 inhibits Akt phosphorylation in C2C12 myoblasts]." Sheng Wu Gong Cheng Xue Bao **25**(10): 1524-31.

Zhang, J., J. C. Grindley, et al. (2006). "PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention." Nature **441**(7092): 518-22.

Zhang, T. T., H. Li, et al. (2009). "Phosphoinositide 3-kinase-regulated adapters in lymphocyte activation." Immunol Rev **232**(1): 255-72.

Zhang, W. C., N. Shyh-Chang, et al. (2012). "Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis." Cell **148**(1-2): 259-72.

Zhang, X., T. Chen, et al. (2012). "Notch1 promotes glioma cell migration and invasion by stimulating beta-catenin and NF-kappaB signaling via AKT activation." Cancer Sci **103**(2): 181-90.

Zhao, Z., Y. Liu, et al. (2011). "Candidate genes influencing sensitivity and resistance of human glioblastoma to Semustine." Brain Res Bull **86**(3-4): 189-94.

Zhou, B. P., Y. Liao, et al. (2001). "Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells." Nat Cell Biol **3**(3): 245-52.

Zhou, J., J. Wulfkuhle, et al. (2007). "Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance." Proc Natl Acad Sci U S A **104**(41): 16158-63.

Zhou, S., J. D. Schuetz, et al. (2001). "The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype." Nat Med **7**(9): 1028-34.

Ziera, T., H. Irlbacher, et al. (2009). "Cnksr3 is a direct mineralocorticoid receptor target gene and plays a key role in the regulation of the epithelial sodium channel." FASEB J **23**(11): 3936-46.

Zimmermann, S. and K. Moelling (1999). "Phosphorylation and regulation of Raf by Akt (protein kinase B)." Science **286**(5445): 1741-4.

Zinzalla, V., D. Stracka, et al. (2011). "Activation of mTORC2 by association with the ribosome." Cell **144**(5): 757-68.

Ziv-Av, A., D. Taller, et al. (2011). "RTVP-1 expression is regulated by SRF downstream of protein kinase C and contributes to the effect of SRF on glioma cell migration." Cell Signal **23**(12): 1936-43.

Zoll, W. L., L. E. Horton, et al. (2002). "Characterization of mammalian eIF2A and identification of the yeast homolog." J Biol Chem **277**(40): 37079-87.

Zoncu, R., A. Efeyan, et al. (2011). "mTOR: from growth signal integration to cancer, diabetes and ageing." Nat Rev Mol Cell Biol **12**(1): 21-35.

Appendix 1: Statistic analysis of significance of the effects of Wortmannin (W) combined with Rapamycin (R) on cells

1. Jurkat T (1)

W	10 nMW	100 nMW	1 μMW	4 μMW
W+R	10 nMW+1 μMR	100 nMW+1 μMR	1 μMW+1 μMR	4 μMW+1 μMR
P-value	0.0304	0.0304	0.0304	0.168

R	1 μMR	1 μMR	1 μMR	1 μMR
W+R	10 nMW+1 μMR	100 nMW+1 μMR	1 μMW+1 μMR	4 μMW+1 μMR
P-value	0.402	0.281	0.000	0.0304

R	1 μMR	1 μMR	1 μMR	1 μMR
W	10 nMW	100 nMW	1 μMW	4 μMW
P-value	0.0304	0.0304	0.0304	0.002

2. Jurkat T (2)

W	4 μMW	4 μMW	4 μMW	4 μMW
W+R	4 μMW+10 nMR	4 μMW+100 nMR	4 μMW+500 nMR	4 μMW+2.5 μMR
P-value	0.0304	0.000	0.0304	0.0304

R	10 nMR	100 nMR	500 nMR	2.5 µMR
W+R	4 µMW+10 nMR	4 µMW+100 nMR	4 µMW+500 nMR	4 µMW+2.5 µMR
P-value	0.0304	0.0304	0.0304	0.000

R	10 nMR	100 nMR	500 nMR	2.5 µMR
W	4 µMW	4 µMW	4 µMW	4 µMW
P-value	0.001	0.002	0.004	0.065

3. 3132 (1)

W	10 nMW	100 nMW	1 µMW	10 µMW
W+R	10 nMW+2.5 µMR	100 MW+2.5 µMR	1 µMW+2.5 µMR	10 µMW+2.5 µMR
P-value	0.0304	0.0304	0.002	0.0304

R	2.5 µMR	2.5 µMR	2.5 µMR	2.5 µMR
W+R	10 nMW+2.5 µMR	100 MW+2.5 µMR	1 µMW+2.5 µMR	10 µMW+2.5 µMR
P-value	0.104	0.113	0.003	0.000

R	2.5 µMR	2.5 µMR	2.5 µMR	2.5 µMR
W	10 nMW	100 nMW	1 µMW	10 µMW
P-value	0.0304	0.0304	0.0304	0.001

4. 3132 (2)

W	8 μMW	8 μMW	8 μMW	8 μMW
W+R	8 μMW+10 nMR	8 μMW+100 nMR	8 μMW+500 nMR	8 μMW+2.5 μMR
P-value	0.0304	0.0304	0.0304	0.0304

R	10 nMR	100 nMR	500 nMR	2.5 μMR
W+R	8 μMW+10 nMR	8 μMW+100 nMR	8 μMW+500 nMR	8 μMW+2.5 μMR
P-value	0.0304	0.0304	0.001	0.000

R	10 nMR	100 nMR	500 nMR	2.5 μMR
W	8 μMW	8 μMW	8 μMW	8 μMW
P-value	0.003	0.002	0.02	0.003

5. J3T (1)

W	100 nMW	1 μMW	10 μMW	20 μMW
W+R	100 nMW+10 μMR	1 μMW+10 μMR	10 μMW+10 μMR	20 μMW+10 μMR
P-value	0.0304	0.0304	0.0304	0.0304

R	10 μMR	10 μMR	10 μMR	10 μMR
W+R	100 nMW+10 μMR	1 μMW+10 μMR	10 μMW+10 μMR	20 μMW+10 μMR
P-value	0.725	0.204	0.213	0.007

R	10 μMR	10 μMR	10 μMR	10 μMR
W	100 nMW	1 μMW	10 μMW	20 μMW
P-value	0.0304	0.0304	0.0304	0.000

6. J3T (2)

W	16 μMW	16 μMW	16 μMW	16 μMW
W+R	16 μMW+10 nMR	16 μMW+100 nMR	16 μMW+1 μMR	16 μMW+10 μMR
P-value	0.0304	P=0.0304	P=0.000	P=0.0304

R	10 nMR	100 nMR	1 μMR	10 μMR
W+R	16 μMW+10 nMR	16 μMW+100 nMR	16 μMW+1 μMR	16 μMW+10 μMR
P-value	0.003	0.006	0.031	0.002

R	10 nMR	100 nMR	1 μMR	10 μMR
W	16 μMW	16 μMW	16 μMW	16 μMW
P-value	0.001	0.000	0.000	0.0304

7. SB (1)

W	100 nMW	1 μMW	10 μMW	20 μMW
W+R	100 nMW+10 μMR	1 μMW+10 μMR	10 μMW+10 μMR	20 μMW+10 μMR
P value	0.001	0.0304	0.0304	0.0304

R	10 µMR	10 µMR	10 µMR	10 µMR
W+R	100 nMW+10 µMR	1 µMW+10 µMR	10 µMW+10 µMR	20 µMW+10 µMR
P-value	0.033	0.007	0.0304	0.0304

R	10 µMR	10 µMR	10 µMR	10 µMR
W	100 nMW	1 µMW	10 µMW	20 µMW
P value	0.0304	0.0304	0.0304	0.004

8. SB (2)

W	16 µMW	16 µMW	16 µMW	16 µMW
W+R	16 µMW+10 nMR	16 µMW+100 nMR	16 µMW+1 µMR	16 µMW+10 µMR
P-value	0.000	0.0304	0.000	0.0304

R	10 nMR	100 nMR	1 µMR	10 µMR
W+R	16 µMW+10 nMR	16 µMW+100 nMR	16 µMW+1 µMR	16 µMW+10 µMR
P-value	0.000	0.0304	0.001	0.0304

R	10 nMR	100 nMR	1 µMR	10 µMR
W	16 µMW	16 µMW	16 µMW	16 µMW
P-value	0.020	0.037	0.039	0.193

9. C2 (1)

W	10 nMW	100 nMW	1 μMW	4 μMW
W+R	10 nMW+1 μMR	100 nMW+1 μMR	1 μMW+1 μMR	4 μMW+1 μMR
P-value	0.000	0.000	0.001	0.001

R	1 μMR	1 μMR	1 μMR	1 μMR
W+R	10 nMW+1 μMR	100 nMW+1 μMR	1 μMW+1 μMR	4 μMW+1 μMR
P-value	0.699	0.089	0.089	0.0304

R	1 μMR	1 μMR	1 μMR	1 μMR
W	10 nMW	100 nMW	1 μMW	4 μMW
P-value	0.000	0.0304	0.0606	0.955

10. C2 (2)

W	4 μMW	4 μMW	4 μMW	4 μMW
W+R	10 nMR+4 μMW	100 nMR+4 μMW	500 nMR+4 μMW	2.5 μMR+4 μMW
P-value	0.001	0.001	0.001	0.0304

R	10 nMR	100 nMR	500 nMR	2.5 μMR
W+R	10 nMR+4 μMW	100 nMR+4 μMW	500 nMR+4 μMW	2.5 μMR+4 μMW
P-value	0.0304	0.0304	0.0304	0.0304

R	10 nMR	100 nMR	500 nMR	2.5 μMR
W	4 μMW	4 μMW	4 μMW	4 μMW
P-value	0.003	0.001	0.205	0.059

11. REM134 (1)

W	100 nMW	1 μMW	10 μMW	20 μMW
W+R	100 nMW+10 μMR	1 μMW+10 μMR	10 μMW+10 μMR	20 μMW+10 μMR
P-value	0.0304	0.0304	0.0304	0.0304

R	10 μMR	10 μMR	10 μMR	10 μMR
W+R	100 nMW+10 μMR	1 μMW+10 μMR	10 μMW+10 μMR	20 μMW+10 μMR
P-value	0.477	0.424	0.1124	0.080

R	10 μMR	10 μMR	10 μMR	10 μMR
W	100 nMW	1 μMW	10 μMW	20 μMW
P-value	0.0304	0.000	0.0304	0.0304

12. REM134 (2)

W	16 μMW	16 μMW	16 μMW	16 μMW
W+R	10 nMR+16 μMW	100 nMR+16 μMW	1 μMR+16 μMW	10 μMR+16 μMW
P-value	0.000	0.000	0.0304	0.0304

R	10 nMR	100 nMR	1 μMR	10 μMR
W+R	10 nMR+16 μMW	100 nMR+16 μMW	1 μMR+16 μMW	10 μMR+16 μMW
P-value	0.994	0.634	0.295	0.053

R	10 nMR	100 nMR	1 μMR	10 μMR
W	16 μMW	16 μMW	16 μMW	16 μMW
P-value	0.000	0.000	0.000	0.0304

Appendix 2: Statistic analysis of significance of the effects of ZSTK474 (Z) combined with Rapamycin (R) on cells

1.Jurkat T

Z	50 nMZ	100 nMZ	250 nMZ	500 nMZ
Z+R	50 nMZ+25 nMR	100 nMZ+25 nMR	250 nMZ+25 nMR	500 nMZ+25 nMR
P-value	0.0809	0.006	0.001	0.0809

R	25 nMR	25 nMR	25 nMR	25 nMR
Z+R	50 nMZ+25 nMR	100 nMZ+25 nMR	250 nMZ+25 nMR	500 nMZ+25 nMR
P value	0.017	0.003	0.000	0.0809

R	25 nMR	25 nMR	25 nMR	25 nMR
Z	50 nMZ	100 nMZ	250 nMZ	500 nMZ
P-value	0.0809	0.028	0.234	0.0809

2. 3132

Z	250 nMZ	400 nMZ	600 nMZ	800 nMZ
Z+R	250 nMZ+5 μMR	400 nMZ+5 μMR	600 nMZ+5 μMR	800 nMZ+5 μMR
P-value	0.0304	0.0304	0.0304	0.0304

R	5 μMR	5 μMR	5 μMR	5 μMR
Z+R	250 nMZ+5 μMR	400 nMZ+5 μMR	600 nMZ+5 μMR	800 nMZ+5 μMR
P-value	0.001	0.000	0.0304	0.0304

R	5 μMR	5 μMR	5 μMR	5 μMR
Z	250 nMZ	400 nMZ	600 nMZ	800 nMZ
P-value	0.000	0.003	0.962	0.281

3. SB

Z	50 nMZ	100 nMZ	250 nMZ	500 nMZ
Z+R	50 nMZ+5 μMR	100 nMZ+5 μMR	250 nMZ+5 μMR	500 nMZ+5 μMR
P-value	0.0809	0.0809	0.0809	0.0809

R	5 μMR	5 μMR	5 μMR	5 μMR
Z+R	50 nMZ+5 μMR	100 nMZ+5 μMR	250 nMZ+5 μMR	500 nMZ+5 μMR
P-value	0.0809	0.017	0.001	0.000

R	5 μMR	5 μMR	5 μMR	5 μMR
Z	50 nMZ	100 nMZ	250 nMZ	500 nMZ
P-value	0.0809	0.0809	0.002	0.087

4. REM134

Z	100 nMZ	500 nMZ	1 μMZ	4 μMZ
Z+R	100 nMZ+1 μMR	500 nMZ+1 μMR	1 μMZ+1 μMR	4 μMZ+1 μMR
P-value	0.0809	0.0809	0.0809	0.001

R	1 μMR	1 μMR	1 μMR	1 μMR
Z+R	100 nMZ+1 μMR	500 nMZ+1 μMR	1 μMZ+1 μMR	4 μMZ+1 μMR
P-value	1.000	0.0809	0.0809	0.0809

R	1 μMR	1 μMR	1 μMR	1 μMR
Z	100 nMZ	500 nMZ	1 μMZ	4 μMZ
P-value	0.0809	0.0809	0.0809	0.000

5. J3T

Z	250 nMZ	500 nMZ	750 nMZ	1 μMZ
Z+R	250 nMZ+1 μMR	500 nMZ+1 μMR	750 nMZ+1 μMR	1 μMZ+1 μMR
P-value	0.004	0.001	0.001	0.001

R	1 μMR	1 μMR	1 μMR	1 μMR
Z+R	250 nMZ+1 μMR	500 nMZ+1 μMR	750 nMZ+1 μMR	1 μMZ+1 μMR
P-value	0.008	0.000	0.000	0.0809

R	1 μMR	1 μMR	1 μMR	1 μMR
Z	250 nMZ	500 nMZ	750 nMZ	1 μMZ
P-value	0.099	0.664	0.034	0.001

6. C2

Z	250 nMZ	500 nMZ	750 nMZ	1 μMZ
Z+R	250 nMZ+500 nMR	500 nMZ+500 nMR	750 nMZ+500 nMR	1 μMZ+500 nMR
P-value	0.000	0.0304	0.000	0.0304

R	500 nMR	500 nMR	500 nMR	500 nMR
Z+R	250 nMZ+500 nMR	500 nMZ+500 nMR	750 nMZ+500 nMR	1 μMZ+500 nMR
P-value	0.0304	0.0304	0.0304	0.0304

R	500 nMR	500 nMR	500 nMR	500 nMR
Z	250 nMZ	500 nMZ	750 nMZ	1 μMZ
P-value	0.396	0.006	0.001	0.0304

Appendix 3: Statistic analysis of significance of the effects of Doxorubicin (D) combined with Wortmannin (W), ZSTK474 (Z), KP372-1 (K), or Rapamycin (R) on cells

1. SB: Doxorubicin (D) and Wortmannin (W)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+W	2.5 nMD+20 μMW	5 nMD+20 μMW	10 nMD+20 μMW	15 nMD+20 μMW
P-value	0.0809	0.0809	0.000	0.0809

W	20 μMW	20 μMW	20 μMW	20 μMW
D+W	2.5 nMD+20 μMW	5 nMD+20 μMW	10 nMD+20 μMW	15 nMD+20 μMW
P-value	0.007	0.003	0.001	0.0809

D	2.5 nMD	5 nMD	10 nMD	15 nMD
W	20 μMW	20 μMW	20 μMW	20 μMW
P-value	0.0809	0.000	0.008	0.41

2. SB: Doxorubicin (D) and ZSTK474 (Z)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+Z	2.5 nMD+100 nMZ	5 nMD+100 nMZ	10 nMD+100 nMZ	15 nMD+100 nMZ
P value	0.001	0.001	0.015	0.0809

Z	100 nMZ	100 nMZ	100 nMZ	100 nMZ
D+Z	2.5 nMD+100 nMZ	5 nMD+100 nMZ	10 nMD+100 nMZ	15 nMD+100 nMZ
P-value	0.481	0.455	0.078	0.002

D	2.5 nMD	5 nMD	10 nMD	15 nMD
Z	100 nMZ	100 nMZ	100 nMZ	100 nMZ
P-value	0.001	0.000	0.030	0.003

3. SB: Doxorubicin (D) and KP372-1 (K)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+K	2.5 nMD+80 nMK	5 nMD+80 nMK	10 nMD+80 nMK	15 nMD+80 nMK
P-value	0.0809	0.0809	0.0809	0.0809

K	80nMK	80nMK	80nMK	80nMK
D+K	2.5 nMD+80 nMK	5 nMD+80 nMK	10 nMD+80 nMK	15 nMD+80 nMK
P-value	0.008	0.0809	0.0809	0.0809

D	2.5 nMD	5 nMD	10 nMD	15 nMD
K	80 nMK	80 nMK	80 nMK	80 nMK
P-value	0.000	0.000	0.001	0.000

4. SB: Doxorubicin (D) and Rapamycin (R)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+R	2.5 nMD+5 μMR	5 nMD+5 μMR	10 nMD+5 μMR	15 nMD+5 μMR
P-value	0.0809	0.001	0.0809	0.002

R	5 μMR	5 μMR	5 μMR	5 μMR
D+R	2.5 nMD+5 μMR	5 nMD+5 μMR	10 nMD+5 μMR	15 nMD+5 μMR
P-value	0.0809	0.041	0.001	0.0809

D	2.5 nMD	5 nMD	10 nMD	15 nMD
R	5 μMR	5 μMR	5 μMR	5 μMR
P-value	0.0809	0.002	0.001	0.637

5. REM134: Doxorubicin (D) and Wortmannin (W)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+W	2.5 nMD+20 μMW	5 nMD+20 μMW	10 nMD+20 μMW	15 nMD+20 μMW
P-value	0.539	0.782	0.450	0.096

W	20 μMW	20 μMW	20 μMW	20 μMW
D+W	2.5 nMD+20 μMW	5 nMD+20 μMW	10 nMD+20 μMW	15 nMD+20 μMW
P-value	0.068	0.033	0.194	0.045

D	2.5 nMD	5 nMD	10 nMD	15 nMD
W	20 μMW	20 μMW	20 μMW	20 μMW
P-value	0.018	0.065	0.069	0.322

6. REM134: Doxorubicin (D) and ZSTK474 (Z)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+Z	2.5 nMD+1 μMZ	5 nMD+1 μMZ	10 nMD+1 μMZ	15 nMD+1 μMZ
P-value	0.001	0.001	0.001	0.008

Z	1 μMZ	1 μMZ	1 μMZ	1 μMZ
D+Z	2.5 nMD+1 μMZ	5 nMD+1 μMZ	10 nMD+1 μMZ	15 nMD+1 μMZ
P-value	0.640	0.264	0.707	0.639

D	2.5 nMD	5 nMD	10 nMD	15 nMD
Z	1 μMZ	1 μMZ	1 μMZ	1 μMZ
P-value	0.001	0.001	0.001	0.008

7. REM: Doxorubicin (D) and KP372-1 (K)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+K	2.5 nMD+5 nMK	5 nMD+5 nMK	10 nMD+5 nMK	15 nMD+5 nMK
P value	0.000	0.004	0.060	0.045

K	5 nMK	5 nMK	5 nMK	5 nMK
D+K	2.5 nMD+5 nMK	5 nMD+5 nMK	10 nMD+5 nMK	15 nMD+5 nMK
P-value	0.326	0.745	0.988	0.200

D	2.5 nMD	5 nMD	10 nMD	15 nMD
K	5 nMK	5 nMK	5 nMK	5 nMK
P-value	0.002	0.004	0.036	0.3827

8. REM134: Doxorubicin (D) and Rapamycin (R)

D	2.5 nMD	5 nMD	10 nMD	15 nMD
D+R	2.5 nMD+5 μMR	5 nMD+5 μMR	10 nMD+5 μMR	15 nMD+5 μMR
P-value	0.001	0.001	0.0809	0.000

R	5 μMR	5 μMR	5 μMR	5 μMR
D+R	2.5 nMD+5 μMR	5 nMD+5 μMR	10 nMD+5 μMR	15 nMD+5 μMR
P-value	0.756	0.593	0.073	0.015

Doxo	2.5 nMD	5 nMD	10 nMD	15 nMD
Rapa	5 μMR	5 μMR	5 μMR	5 μMR
P-value	0.001	0.001	0.000	0.004

9. 3132: Doxorubicin (D) + Wortmannin (W)

D	2 nMD	4 nMD	6 nMD	8 nMD
D+W	2 nMD+8 μMW	4 nMD+8 μMW	6 nMD+8 μMW	8 nMD+8 μMW
P-value	0.005	0.007	0.250	0.684

W	8 μMW	8 μMW	8 μMW	8 μMW
D+W	2 nMD+8 μMW	4 nMD+8 μMW	6 nMD+8 μMW	8 nMD+8 μMW
P-value	0.049	0.001	0.0809	0.0809

D	2 nMD	4 nMD	6 nMD	8 nMD
W	8 μMW	8 μMW	8 μMW	8 μMW
P-value	0.291	0.003	0.000	0.0809

10. 3132: Doxorubicin (D) + ZSTK474 (Z)

D	2 nMD	4 nMD	6 nMD	8 nMD
D+Z	2 nMD+500 nMZ	4 nMD+500 nMZ	6 nMD+500 nMZ	8 nMD+500 nMZ
P-value	0.0809	0.015	0.021	0.000

Z	500 nMZ	500 nMZ	500 nMZ	500 nMZ
D+Z	2 nMD+500 nMZ	4 nMD+500 nMZ	6 nMD+500 nMZ	8 nMD+500 nMZ
P-value	0.013	0.006	0.001	0.000

D	2 nMD	4 nMD	6 nMD	8 nMD
Z	500 nMZ	500 nMZ	500 nMZ	500 nMZ

P-value	0.000	0.069	0.000	0.0809

11. 3132: Doxorubicin (D) + Rapamycin (R)

D	2 nMD	4 nMD	6 nMD	8 nMD
D+R	2 nMD+40 nMR	4 nMD+40 nMR	6 nMD+40 nMR	8 nMD+40 nMR
P-value	0.003	0.005	0.735	0.000

R	40 nMR	40 nMR	40 nMR	40 nMR
D+R	2 nMD+40 nMR	4 nMD+40 nMR	6 nMD+40 nMR	8 nMD+40 nMR
P-value	0.565	0.081	0.013	0.011

D	2 nMD	4 nMD	6 nMD	8 nMD
R	40 nMR	40 nMR	40 nMR	40 nMR
P-value	0.013	0.158	0.017	0.002

12. C2: Doxorubicin (D) and Wortmannin (W)

D	5 nMD	10 nMD	15 nMD	20 nMD
D+W	5 nMD+8 μMW	10 nMD+8 μMW	15 nMD+8 μMW	20 nMD+8 μMW
P-value	0.013	0.158	0.591	0.003

W	8 μMW	8 μMW	8 μMW	8 μMW
D+W	5 nMD+8 μMW	10 nMD+8 μMW	15 nMD+8 μMW	20 nMD+8 μMW

P-value	0.434	0.020	0.008	0.002

D	5 nMD	10 nMD	15 nMD	20 nMD
W	8 μMW	8 μMW	8 μMW	8 μMW
P-value	0.047	0.032	0.009	0.009

13. C2: Doxorubicin (D) and ZSTK474 (Z)

D	5 nMD	10 nMD	15 nMD	20 nMD
D+Z	5 nMD+1 μMZ	10 nMD+1 μMZ	15 nMD+1 μMZ	20 nMD+1 μMZ
P-value	0.0809	0.005	0.002	0.101

Z	1 μMZ	1 μMZ	1 μMZ	1 μMZ
D+Z	5 nMD+1 μMZ	10 nMD+1 μMZ	15 nMD+1 μMZ	20 nMD+1 μMZ
P-value	0.423	0.015	0.006	0.007

Doxo	5 nMD	10 nMD	15 nMD	20 nMD
ZSTK	1 μMZ	1 μMZ	1 μMZ	1 μMZ
P-value	0.0809	0.011	0.3827	0.543

14. C2: Doxorubicin (D) and Rapamycin (R)

D	5 nMD	10 nMD	15 nMD	20 nMD
D+R	5 nMD+8 μMR	10 nMD+8 μMR	15 nMD+8 μMR	20 nMD+8 μMR
P-value	0.000	0.003	0.760	0.050

R	8 μMR	8 μMR	8 μMR	8 μMR
D+R	5 nMD+8 μMR	10 nMD+8 μMR	15 nMD+8 μMR	20 nMD+8 μMR
P-value	0.080	0.008	0.019	0.006

Doxo	5 nMD	10 nMD	15 nMD	20 nMD
Rapa	8 μMR	8 μMR	8 μMR	8 μMR
P value	0.001	0.286	0.017	0.004

Appendix 4: Table for glioma-specific up-regulated and down-regulated genes

Symbol	Fold Change	Function	Importance in gliomas
Activator of class I PI3K			
DDR1	1.673	DDR1 encodes discoidin domain receptor-1 (DDR1), which is a type of RTK that can activate its downstream effectors, including class I PI3K and Raf MAPK pathways, in response to stimulation of growth factors (Marshall 1995; Lemmon and Schlessinger 2010)	DDR1 is commonly expressed in all malignant central nervous system (CNS) human brain tumours, including glioblastoma (Weiner *et al.* 2000). DDR1 has a role in stimulate invasion and adhesion of human glioma cells (Ram *et al.* 2006).
FGFR1	1.920	FGFR1 encodes fibroblast growth factor receptor 1 (FGFR1), which is a type of RTK that can activate its downstream effectors, including class I PI3K and Raf MAPK pathways, in response to stimulation of growth factors (Marshall 1995; Lemmon and Schlessinger 2010).	FGFR1 plays a key role in promoting the growth and angiogenesis of glioma cells (Yamada *et al.* 1998; Yamada *et al.* 1999; Auguste *et al.* 2001).
FGFR2	-2.200	FGFR2 encodes fibroblast growth factor receptor 2 (FGFR2), which is a type of RTK that can activate its downstream effectors, including class I PI3K and Raf MAPK pathways, in response to stimulation of growth factors (Marshall 1995; Lemmon and Schlessinger 2010).	FGFR2 was reported to promote growth and angiogenesis in C6 glioma cells (Auguste *et al.* 2001). Paradoxically, this RTK exerted no effect on the growth of other malignant glioma cell lines (Yamada *et al.* 1999). It is suggested that FGFR2-modulated tumour progression is cell line-specific.
FLT1	-2.106	FLT1 encodes Fms-like	In human astrocytoma

		tyrosine kinase 1 (Flt1), also named VEGFR1, which is a type of RTK that can activate its downstream effectors, including class I PI3K and Raf MAPK pathways, in response to stimulation of growth factors (Marshall 1995; Lemmon and Schlessinger 2010). Flt1 acts to promote angiogenesis after binding to its ligand VEGF (Xiang *et al.* 2001).	specimens, both tumour and endothelial cells expressed similar levels of Flt1. Besides, high grade astrocytomas expressed higher levels of Flt1 than low-grade astrocytomas (Xiang *et al.* 2001). This protein acts to promote angiogenesis in astrocytoma (Xiang *et al.* 2001).
FYN	9.334	FYN encodes protein-tyrosine kinase Fyn, which belongs to Src protein family. Once RTK activates Fyn, it can subsequently activate class I PI3K (Saito *et al.* 2010). Fyn promotes oligodendroglial cell differentiation and myelin formation in CNS in response to apotransferrin stimulation (Perez *et al.* 2009).	Overexpression of Fyn was observed in many human cancers, such as glioma and leukemia, and was associated with promoting tumour invasion and migration (Yadav and Denning 2011). Fyn was reported to phosphorylate CD133 (a stem cell marker) in human medulloblastoma cells, suggesting playing a role in regulation of CD133 (Boivin *et al.* 2009).
GAB1	2.444	GAB1 encodes Gab1, which is an adaptor protein. Gab1 can activate class I PI3K after Gab1 is phosphorylated by activated RTK (Rodrigues *et al.* 2000).	Down-regulation of Gab1 expression was reported in low grade human astrocytoma specimens (Huang *et al.* 2000). In high grade glioblastoma cells, the Shp-2 phosphatase/Gab1 complex activates class I PI3K/Akt/NF-κB signaling after EGFR activation (Kapoor *et al.* 2004).
GHR	1.426	GHR encodes growth hormone receptor (GHR), which can activate class I PI3K through activation of GHR/JAK2/IRS-1 signaling (Herrington and	Expression of GHR was detected in human CNS tissue and the human gliomblastoma U87MG cell line (Castro *et al.* 2000).

		Carter-Su 2001).	
GRB2	-1.190	GRB2 encodes growth factor receptor-bound protein 2 (Grb2), which acts as an adaptor protein of RTK. The interaction between active RTK and Grb2 promotes Grb2 to activate class I PI3K through Gab1 or Ras (Cully *et al.* 2006).	Grb2, along with Ras and Shc, played a role in up-regulating tumourigeneity in human glioblastoma cells expressing mutant DeltaEGFR expression (Prigent *et al.* 1996).
IGF1R	-1.554	IGF1R encodes IGF-1R, which is a type of RTK. The IGF-1R is often activated by its ligands, IGF-1 and IGF-2. The IGF-1R exerts the growth effect on normal and cancer cells by activation of its downstream class I PI3K/Akt and Raf1 MAPK signaling (Pollak 2012).	IGF1R was reported to positively regulate the growth of glioblastoma (Hagerstrand *et al.* 2010). Besides, genetic variation in IGF1R was reported to be correlated with low grade glioma but not high grade glioblastoma (Lonn *et al.* 2008).
INSR	1.747	INSR encodes IR, which is a type of RTK. IR contains A isoform (IR-A) and B isoform (IR-B). IR is activated by its ligand insulin. IR-A can also be activated by IGF-2. The main downstream pathways of IR are class I PI3K/Akt and Raf MAPK pathways. IR plays a key role in regulate carbohydrate metabolism in both normal and tumour cells (Marshall 1995; Lemmon and Schlessinger 2010; Belfiore and Malaguarnera 2011).	Insulin was reported to up-regulate proliferation of low grade gliomas, promote differentiation of high grade glioblastoma, and increase glucose uptake of glioma cells (Grunberger *et al.* 1986; Glick *et al.* 1989).
IRS1	1.292	IRS1 encodes IRS-1, a RTK adaptor protein. IRS-1 acts to activate PI3K after the interaction of IRS-1 and RTK (Wymann and Pirola 1998).	The level of IRS-1 was positively correlated with higher grade of human gliomas (Qian *et al.* 2008).
JAK2	2.405	JAK2 encodes Jak2 which activates Stat, Grb2/Erk and IRS-1/class I PI3K pathways after Jak2 is activated by its	Constitutive activation of Jak2/Stat3 was observed to contribute to overgrowth of the human GL15 glioblastoma cell

		regulators, such as growth hormone and cytokine receptors (VanderKuur *et al.* 1995; Leonard and O'Shea 1998; Herrington and Carter-Su 2001).	line (Sciaccaluga *et al.* 2007). A recent study showed that Jak2/Stat3 pathway positively regulated invasion and migration of human glioblastoma (Senft *et al.* 2011). Jak2/Stat3 pathway has a role in up-regulating proliferation and survival of glioblastoma stem cells (Sai *et al.* 2012).
KDR	1.764	KDR encodes vascular endothelial growth factor receptor 2 (VEGFR2), which is a type of RTK that can activate its downstream effectors, including class I PI3K and Raf MAPK pathways, in response to stimulation of growth factors (Marshall 1995; Lemmon and Schlessinger 2010).	Constitutive activation of VEGFR2 was frequently observed in high grade astrocytomas but not in low grade gliomas (Carroll *et al.* 1999). Autocrine regulation and expression of VEGFR2 and its ligand VEGF was positively correlated with the radioresistance of glioblastoma cells (Geng *et al.* 2001; Knizetova *et al.* 2008). However, there is a debate on the roles of VEGFR2 and VEGF in regulation of the proliferation of glioma cells (Hong *et al.* 2007; Knizetova *et al.* 2008).
KRAS	-2.151	KRAS encodes K-Ras GTPase, which acts as an upstream activator of both class I PI3K and Raf MAPK pathways (Castellano and Downward 2011).	Expression of K-ras or constitutive activation of mutant K-Ras was observed to promote gliomagenesis in human patient and mouse model (Holmen and Williams 2005; Janzarik *et al.* 2007).
NRAS	-1.288	NRAS encodes N-Ras GTPase, which acts as an upstream activator of both class I PI3K and Raf MAPK pathways (Castellano and Downward 2011).	N-Ras with acting mutation is responsible for initiating certain human glioblastomas (Knobbe *et al.* 2004).

NTRK2	7.805	NTRK2 encoding TrkB RTK, which activates its downstream pathways, including Ras/Raf, class I PI3K/Akt, PLCγ, NF-κB, atypical PKC (ζ and ι) in response to BDNF, and neurotrophin 4 and 5 (NT4 and NT5) (Marshall 1995; Huang and Reichardt 2003; Lemmon and Schlessinger 2010). TrkB is ubiquitously expressed on all the cells of nervous system. An *in vivo* experiment showed that TrkB receptor was critical for the viability of sensory and motor neurons in mouse model (Barbacid 1994).	Expression of NTRK2 was frequently detected in human astrocytic gliomas (Wang *et al.* 1998). Expression of truncated NTRK2 was reported to regulate RhoA-modulated cell morphology and cytoskeletal rearrangement in rat C6 glioma cell line in response to BDNF (Ohira *et al.* 2006). NTRK2 expressed two forms of TrkB, truncated and full-length TrkB. The truncated TrkB was frequently expressed in neural stem cells and glial cells whereas full-length TrkB was expressed in mature neurons (Islam *et al.* 2009). The BDNF/TrkB signaling plays a role in promoting proliferation, differentiation and migration and guiding direction of embryonic and adult neural stem cells/progenitors (Tervonen *et al.* 2006; Bartkowska *et al.* 2007; Islam *et al.* 2009; Bagley and Belluscio 2010).
NTRK3	3.138	NTRK3encodes TrkC RTK, which activates its downstream pathways, including Ras/Raf, class I PI3K/Akt, PLCγ, NF-κB, atypical PKC (ζ and ι) in response to NT3 (Huang and Reichardt 2003). TrkC is ubiquitously expressed on all the cells of nervous system and some non-nerve system structures, such as tooth and diaphram. TrkC-null mouse model resulted in completely loss of la muscle afferents at spinal cord and partially loss of	Expression of NTRK3 was frequent in human astrocytic gliomas (Wang *et al.* 1998). NT3/TrkC signaling regulates neural cell development and differentiation (Klopotowska and Strzadala 2005; Wang *et al.* 2007).

		neurons, axons and nerve fiber (Barbacid 1994).	
PDGFC	1.743	PDGFC encodes platelet derived growth factor C (PDGF-C). The PDGF-C frequently forms a homodimer, thus becoming PDGF-CC. The PDGF-CC can activate class I PI3K signaling through binding to PDGF receptor (PDGFR) αα (PDGFR αα) or PDGFR αβ (Calzolari and Malatesta 2010).	PDGF-C is abundantly expressed in gliomas but is rarely found in normal brain tissue (Lokker *et al.* 2002). PDFG-C functioned in promoting vascular maturation in gliomas (di Tomaso *et al.* 2009).
PDGFRA	2.371	PDGFRA encodes PDGF receptor α (PDGFRα). The PDGFRα can either homodimerze with another PDGFRα to form PDGFRαα, or heterodimerize with PDGFβ to form PDGFR αβ. The PDGFR αα and PDGFR αβ belongs to RTK kinase family, which can activates class I PI3K in response to PDGF-AA, AB, BB or CC (Calzolari and Malatesta 2010; Lemmon and Schlessinger 2010).	PDGFRA is commonly expressed in glioma, especially in malignant glioblastomas (Furnari *et al.* 2007). The established autocrine/paracrine regulation of PDGFRs and their ligands may contribute to gliomagenesis (Calzolari and Malatesta 2010). PDGF-A/PDGFRα signaling regulates proliferation and survival of glial progenitors, the differentiation from glial progenitors into oligodendrocytes, and generation of myelin in CNS (Calzolari and Malatesta 2010).
PDGFRB	7.251	PDGFRB encodes PDGF receptor β (PDGFR β). The PDGFR β can either homodimerze with another PDGFR β to form PDGFR ββ, or heterodimerize with PDGFα to form P signaling is associated with PDGFR αβ. The PDGFR ββ and PDGFR αβ belongs to RTK kinase family, which can activates class I PI3K in response to PDGF- AB, BB or DD (Calzolari and Malatesta	PDGFRB is abundantly expressed in endothelial cells in high grade glioblastoma (Furnari *et al.* 2007).

		2010; Lemmon and Schlessinger 2010).	
PTK2	-2.200	PTK2 encodes FAK, a cytoplasmic tyrosine kinase. FAK can activate class I PI3K after FAK is associated with its main upstream activator β-integrin. The β-integrin/FAK signaling regulates cell-cell adhesion and cell movement (Guan 2010).	FAK was abundantly expressed in high-grade human glioblastomas and is involved in positively regulating proliferation, survival, motility and invasion in gliomas (Natarajan *et al.* 2003).
RRAS2	-1.403	Oncogene RRAS2 encodes TC21 (also named R-Ras2). TC21 induces transformation and maintains survival through activation of its downstream pathways including class I PI3K and NF-κB (Rong *et al.* 2002).	N/A
SHC1	1.098	SHC1 encodes ShcA which mainly activates MAPK and c-Myc pathways and cell survival. Alternatively, ShcA associates with Grb2 and subsequently activates class I PI3K in response to IGF-1 stimulation (Ravichandran 2001; Radhakrishnan *et al.* 2008).	Mutant DeltaEGFR was reported to up-regulate tumourigenesis of human glioblastomas through constitutive phosphorylation of its adaptor proteins Shc and Grb2. The phosphorylayed Shc and Grb2 by DeltaEGFR frequently resulted in activation of Ras pathway (Prigent *et al.* 1996).
CBL	5.566	CBL encodes c-Cbl which acts as an adaptor protein and an E3 ubiquitin ligase. c-Cbl can bind to p85 subunit of class IA PI3K and subsequently activate class IA PI3K after tyrosine phosphorylation of c-Cbl by Fyn or Bcr/Abl (Jain *et al.* 1997; Hunter *et al.* 1999).	Wild-type c-Cbl promotes glioma invasion through increasing expression of matrix matrix metalloprotease 2 (MMP2) (Lee and Tsygankov 2010).
PTPN11	-2.548	PTPN11 encodes Shp-2, which positively regulates class I PI3K activation (Wu *et al.* 2001).	Shp-2 acts as a dual regulator of gliomagenesis. On one hand, Shp-2 promote gliomagenesis through

			inhibiting cell aging or relaying signals from RTKs to their downstream pathways such as class I PI3K and Erk (Zhan and O'Rourke 2004; Charest *et al.* 2006; Zhan *et al.* 2009; Liu *et al.* 2011; Sturla *et al.* 2011). On the other hand, this protein binds to the SIRPα1 receptor, which results in inhibiting wild –type EGFR and mutant EGFRvIII-mediated transformation and migration of glioma (Wu *et al.* 2000; Kapoor and O'Rourke 2010).
Inhibitor of class I PI3K			
IGFBP2	27.128	IGFBP2 encodes IGF-binding protein (IGFBP)-2 (IGFBP2), which binds to IGF and subsequently prevents the interaction of IGF with IGF receptor, leading to inhibition of class I PI3K pathway (Rajaram *et al.* 1997). Expression of IGFBP2 mRNA was detected in rat brain at both young age and adulthood. Its expression level was increased with age (Chernausek *et al.* 1993).	High frequency of IGFBP2 overexpression was detected in glioma, especially in high grade human and canine glioblastoma (Becher *et al.* 2008; Lin *et al.* 2009; Stoica *et al.* 2011). The presence of higher IGFBP2 expression levels in blood is positively correlated with poor prognosis (Lin *et al.* 2009). Although IGFBP2 protein in normal cells acts as inhibitor of IGF/class I PI3K signaling, this protein has been reported to be a positive regulator of Akt signaling in PDGFB-induced glioma (Rajaram *et al.* 1997; Dunlap *et al.* 2007). IGFBP2 protein was demonstrated to up-regulate invasion and motility of human GBM cell lines through interacting with integrin-α5 and up-regulating expression of MMP-2 gene. Besides, this protein promoted glioma

			progression through up-regulating CD24 gene expression (Wang *et al.* 2003; Wang *et al.* 2006; Fukushima *et al.* 2007). In pediatric high grade astrocytomas, IGFBP2 promotes malignant phenotype through up-regulating expression of genes encoding several DNA repair enzymes, but not through promoting the invasion and growth of tumour cells (Becher *et al.* 2008). IGFBP2 was demonstrated to induce proliferation, maintain survival and drug resistance of GSCs, in part through Akt signaling (Hsieh *et al.* 2010).
IGFBP4	2.852	IGFBP4 encodes IGFBP4 protein, which binds to IGF and subsequently prevents the interaction of IGF with IGF receptor, leading to inhibition of class I PI3K pathway (Rajaram *et al.* 1997). Expression of IGFBP4 mRNA was detected in rat brain at both young age and adulthood. Its expression level was decreased with age (Chernausek *et al.* 1993).	IGFBP4 has been detected in high-grade human glioblastoma (Schlenska-Lange *et al.* 2008) IGFBP4 was reported to act as tumour suppressor in glioblastoma through down-regulating angiogenesis and tumourigenesis (Moreno *et al.* 2006).
IGFBP6	1.807	IGFBP6 encodes IGFBP6 protein, which binds to IGF and subsequently prevents the interaction of IGF with IGF receptor, leading to inhibition of class I PI3K pathway (Rajaram *et al.* 1997).	IGFBP6 transcript was detected in high grade human glioblastoma (Schlenska-Lange *et al.* 2008).
IGFBP7	2.991	IGFBP7 encodes IGFBP7 protein, which binds to IGF with low affinity and is speculated to negatively	IGFBP7 was specifically expressed in blood vessels of GBM, particularly in endothelial cells and vascular

		regulate IGF/IGF receptor/class I PI3K signaling, based on the findings of lower IGFBP7 levels in cancers than that in normal tissue (Oh *et al.* 1996).	membrane. Conversely, no expression of IGFBP7 was detected in normal brain (Pen *et al.* 2007). Up-regulation of IGFBP7 gene in GBM vessels was found to be stimulated by TGF-β/ALK5/Smad-2 pathway (Pen *et al.* 2008). It was reported that IGFBP7 protein acted as a tumour suppressor in GBM cells through inhibiting cell growth and promoting cell aging. But this protein promoted vascular stabilization in GBM by recruiting smooth muscle cells and inducing trans-differentiation from endothelial cells into vascular smooth muscle cells (Pen *et al.* 2011).
IGF2R	4.862	IGF2R encoding IGFII/mannose-6-phosphate receptor which acts to attenuate IGF-II signaling (Furstenberger and Senn 2002)	Deletion of IGF2R, as a result of chromosomal translocation, was detected in primary human GBM tumours and human GBM cell lines (Mulholland *et al.* 2006).
INPPL1	1.473	INPPL1 encodes Ship-2, which functions in conversion of PtdIns(3,4,5)P3 back to PtdIns(3,4)P2 through dephosphorylation of PtdIns(3,4,5)P3 on the 5' position (Damen *et al.* 1996).	Ship2 inhibited G1/S phase transition and inactivated Akt in glioma cells whereas this positively regulated RhoA-modulated migration and polarization (Taylor *et al.* 2000; Kato *et al.* 2012).
RASA1	1.921	RASA1 encodes RAS p21 protein activator 1 (p120 RasGAP), which has GAP activity toward Ras, leading to inhibition of Ras signaling (Vogel *et al.* 1988; Woodcock and Hughes 2004).	N/A
SOCS2	1.705	SOCS2 encodes suppressor of	SOCS2 expression was

		cytokine signaling (SOCS)-2 (SOCS2), which belongs to SOCS protein family. SOCS family serves as a subunit of E3 ubiquitin ligase which degrades target proteins. In addition, SOCS family functions in inactivation of Jak/Stat pathway (Akhtar and Benveniste 2011). SOCS2 acts as the negative regulator of cytokine/Jak/Stat, growth hormone and IGF-1 pathways (Rico-Bautista *et al.* 2006).	reported to be a key factor for the response of GBM cells to a chemotherapeutic agent Semustine (Zhao *et al.* 2011).
SOCS5	2.045	SOCS5 encodes SOCS5 protein, which belongs to SOCS protein family. SOCS family serves as a subunit of E3 ubiquitin ligase which degrades target proteins. In addition, SOCS family functions in inactivation of Jak/Stat pathway (Akhtar and Benveniste 2011). SOCS5 protein inhibits cytokine IL-4/Jak/Stat and EGFR signaling pathways (Seki *et al.* 2002; Kario *et al.* 2005).	N/A
Activator of Akt			
CSNK2A2	1.357	CSNK2A2 encodes α catalytic subunit of CK2, which is a constitutively active serine/threonine kinase and can phosphorylate its substrates through transferring a phosphate group from ATP and GTP to its substrates. CK2 can activate class I PI3K/Akt signaling pathway through inhibitory phosphorylation of PTEN and active phosphorylation of Akt (Hanif *et al.* 2010). CK2 is associated	CK2 promotes survival and invasion of glioma cells through down-regulation of p53-modulated cell cycle arrest, TNFα-modulated apoptosis and PTEN activity, up-regulation of DNA repair and trans-activation of EGF/Erk signaling-stimulated β-catenin-dependent invasion (Maccario *et al.* 2007; Ji *et al.* 2009; Olsen *et al.* 2010; Dixit *et al.* 2012).

		with promoting proliferation and maintaining survival of tumour cells (Kaminska *et al.* 2009).	
CSNK2A1	-1.226	CSNK2A2 encodes α prime catalytic subunit of CK2 (Hanif *et al.* 2010).	CK2 promotes survival and invasion of glioma cells through down-regulation of p53-modulated cell cycle arrest, TNFα-modulated apoptosis and PTEN activity, up-regulation of DNA repair and trans-activation of EGF/Erk signaling-stimulated β-catenin-dependent invasion (Maccario *et al.* 2007; Ji *et al.* 2009; Olsen *et al.* 2010; Dixit *et al.* 2012).
HSP90B1	-1.393	HSP90B1 encodes heat shock protein 90 kDa (HSP90) beta member 1. The HSP90 acts to assist around 200 proteins to have correct conformation for performing their biological activities, such as cell growth and survival (Messaoudi *et al.* 2008). Among HSP90 client proteins, HSP90 was reported to positively regulate Akt-dependent cell survival and Akt/eNOS signaling-induced angiogenesis (Sato *et al.* 2000; Miyauchi *et al.* 2012).	HSP90 is ubiquitously expressed in gliomas, particularly high-grade glioblastoma, and endothelial cells in glioma vessels (Strik *et al.* 2000). The HSP90 was found to be correlated with tumour progression, including angiogenesis, unlimited DNA replication, invasion, migration, proliferation and survival, via its client proteins (Messaoudi *et al.* 2008). Studies of HSP90 inhibitors highlighted HSP90 played a critical role in G2/M phase transition and maintaining survival in gliomas and the growth of glioma stem cells (Garcia-Morales *et al.* 2007; Sauvageot *et al.* 2009).
MRAS	-1.459	MRAS encodes M-Ras, which belongs to Ras GTPase protein family. M-Ras is commonly	N/A

		expressed in CNS brain tissue and is involved in activation of Akt survival pathway, MAPK-induced neuronal differentiation, integrin pathway and actin cytoskeletal rearrangement (Matsumoto *et al.* 1997; Kimmelman *et al.* 2000; Kimmelman *et al.* 2002; Yoshikawa *et al.* 2007).	
Inhibitor of Akt			
PRKAR1A	1.749	PRKAR1A encodes type 1α regulatory subunit of PKA, which keeps PKA at inactive state (Howe 2004). PKA activates Akt through class I PI3K-independent pathway (Filippa *et al.* 1999).	Dibutyryl cAMP-stimulated PKA activity preferentially increased expression of the type I regulatory subunit but not other subunits of PKA in neuroblastoma-glioma hybrid cells (Lohmann *et al.* 1983). Up-regulation of PKA kinase cascades was found to promote differentiation, induce apoptosis and inhibit growth of high-grade glioblastoma (Chen *et al.* 1998). Type 1α regulatory subunit of PKA counteracts PKA activity (Howe 2004).
PRKAR2A	-1.790	PRKAR2A encodes type 2α regulatory subunit of PKA which keeps PKA at inactive state (Howe 2004). PKA activates Akt through class I PI3K-independent pathway (Filippa *et al.* 1999).	Up-regulation of PKA kinase cascades was found to promote differentiation and apoptosis and growth arrest of high-grade glioblastoma (Chen *et al.* 1998). Type 2α regulatory subunit of PKA counteracts PKA activity (Howe 2004). The type 2 regulatory subunit of PKA is one component of cAMP-stabilizing region (CSR)-protein complex which plays a critical role in mRNA stabilization in rat glioma cells (Jungmann and Kiryukhina

			2005).
THEM4	-2.129	THEM encodes carboxyl-terminal modulator protein (CTMP). The CTMP acts as the negative regulator of Akt (Knobbe *et al.* 2004).	Hypermethylation of CTMP promoter resulting in down-regulation of CTMP mRNA levels is common in glioblastoma (Knobbe *et al.* 2004).
PLD3	3.925	PLD3 encodes phospholipase D (PLD)-3 (PLD3). Unlike the classical PLD1 and PLD2 which produce phosphatidic acid for mTORC1 activation, the function of PLD3 remains unclear (Xu *et al.* 2011). PLD3 was reported to promote cellular differentiation in brain and muscle (Pedersen *et al.* 1998; Osisami *et al.* 2012). Overexpression of PLD3 was found to inactivate Akt signaling in muscle cells (Zhang *et al.* 2009).	N/A
Activator of mTORC1			
PIK3C3	1.619	PIK3C3 encodes class III PI3K which can activate mTORC1 in response to both amino acid and glucose stimulation (Byfield *et al.* 2005; Nobukuni *et al.* 2007).	N/A
Inhibitor of mTORC1			
STK11	1.320	STK11 gene encodes LKB1 which cooperates with AMPK to inhibit mTORC1 signaling when the ratio of intracellular ATP: AMP is low (Oakhill *et al.* 2011).	LKB1/AMPK pathway promotes the migration and survival of glioma cells but arrests tumour cell growth in response to metabolic stress, such as glucose deprivation (Godlewski *et al.* 2010).
DDIT4	8.506	DDIT4 encodes REDD1 which acts as negatively regulates mTORC1 activity in the presence of stress inputs such as hypoxia (Reiling and Hafen	N/A

		2004; Sofer *et al.* 2005).	
PRKAB2	-1.413	PRKAB2 encodes β2 subunit of AMPK which acts as an energy sensor and negatively regulates mTORC1 signaling (Hardie *et al.* 2006).	AMPK attenuates the pro-apoptotic or cytotoxic effect of anti-cancer drugs through induction of autophagy in cancers, including gliomas (Harhaji-Trajkovic *et al.* 2009; Misirkic *et al.* 2012). Another function of AMPK is to promote migration of glioma cells in the presence of gherlin, a gastric peptide (Chen *et al.* 2011). In glioblastomas expressing mutant EGFRVIII, AMPK acts as a tumour suppressor through inhibiting lipogenesis (Guo *et al.* 2009).
Components of class I PI3K/Akt/mTOR axis pathway			
PIK3CD	-2.243	PIK3CD encodes p110δ catalytic subunit of class IA PI3K (Wymann and Pirola 1998)	Overexpression of p110δ was detected in many gliomas *in vitro* and the role of p110δ in glioma was to promote cell migration and invasiveness. Either PIK3CD gene amplification or overexpression of its mRNA in glioblastoma has been documented (Knobbe and Reifenberger 2003; Mizoguchi *et al.* 2004).
PIK3R5	-1.289	PIK3R5 encodes p101 regulatory subunit of class IB PI3K (Voigt *et al.* 2005).	N/A
AKT1	-1.760	AKT1 encodes Akt1 kinase which acts to promote both cellular growth and survival in mouse model (Chen *et al.* 2001; Cho *et al.* 2001). Akt1 was reported to promote motility in normal cells but arrest	Amplification and overexpression of Akt1 gene rarely occurs in glioblastoma (Knobbe and Reifenberger 2003). Although Akt1 kinase contributes to the growth and

		migration in cancer cells.	survival of normal cells in mouse model and the tumour types other than glioma, it has been reported that this protein exerts no effect on the growth and survival of glioma cells *in vitro* (Chen *et al.* 2001; Cho *et al.* 2001; Altomare and Testa 2005; Mure *et al.* 2010).
EIF4E	-1.654	EIF4E encodes eIF4E. This protein participates in triggering translation of capped mRNAs (Mamane *et al.* 2004).	In normal brain tissue, eIF4E was only expressed in pyramid neurons. However, high expression level of eIF4E was detected in malignant glioblastomas (Gu *et al.* 2005). Two reports showed that eIF4E promoted glioma cell growth under normaxia. But this protein sensitized glioma cell to radiation therapy under hypoxic condition, due to increased hypoxia-induced cell death (Dubois *et al.* 2009; Rouschop *et al.* 2011).
Substrates of class I PI3K/Akt/mTOR axis pathway			
ACLY	1.592	ACLY encodes ACL enzyme which participates in glucose-dependent lypogenesis process and is responsible for generation of acetyl- CoA from citrate. The activity of ACL is activated by Akt in response to insulin or growth factor stimulation. The mRNA level of ACL can be up-regulated in response to insulin and is class I PI3K/Akt signaling-dependent (Hatzivassiliou *et al.* 2005).	The migration and invasiveness of glioblastoma cells was reported to depend on ACL-induced glycolysis. High level of ACL was found to be accumulated in pseudopodia (Beckner *et al.* 2010).
CHUK	-1.349	CHUK encodes IKK-α, which acts to dissociate of NF-κB from inhibitors of NF-κB (IκB) through phosphorylation of IκB.	IKK-α is involved in regulation of Notch1/Akt-induced cell migration and invasion (Zhang *et al.* 2012).

		This results in activation of NF-κB transcription factor, which targets genes associated with regulation of immune system, cellular survival, proliferation and differentiation (Hayden and Ghosh 2012).	
FOXO1	2.116	FOXO1 encodes FoxO1, a transcription factor. The FoxO1a can up-regulate or down-regulate its target genes, depending on its binding partner. Akt acts as the negative regulator of FoxO1a in response to the stimulation of growth factors, hormone and cytokines (van der Vos and Coffer 2011).	FoxO1a acts as a tumour suppressor and its activity is often abrogated by active class I PI3K/Akt signaling in gliomas (Ramaswamy *et al.* 2002; Choe *et al.* 2003).
GSK3B	1.381	GSK3B encodes GSK-3β. The GSK-3β maintains neural progenitor at undifferentiated state, inhibits migration and polarization of neurons, and abrogates axon outgrowth through regulation of its downstream targets such as transcription factors and microtubule-associated proteins, and ubiquitin-protease system. Akt and p38MAPK act as negative regulators of GSK-3β (Hur and Zhou 2010).	Phosphorylated GSK-3β on Ser9, indicating inactivation of GSK-3β, was moderately to strongly expressed in a high percentage (71%) of GBM (Otero and Tihan 2011). GSK-3β was reported to induce differentiation of glioblastoma cells (Li *et al.* 2010). In addition, this protein promotes proliferation and maintains survival in glioma through inhibition of Rb and p53 pathways (Miyashita *et al.* 2009). There is conflicting data regarding the role of GSK-3β in glioblastoma stem cells. On one hand, active EGFR/Akt signaling-mediated inhibitory phosphorylation of GSK-3β was found to promote proliferation of neurosphere cells (Howard *et al.* 2010). On the other hand, down-regulation of GSK3B

			expression or GSK-3β activity reduced GBM stem cell pool and induced differentiation and apoptosis (Korur *et al.* 2009).
PRKCI	-1.822	PRKCI encodes atypical protein kinase C iota (PKCι), which promotes cell polarity, proliferation and survival. This protein kinase is activated by PtdIns(3,4,5)P3 and PDK1 (Fields and Regala 2007).	PKCι was reported to promote proliferation, survival and invasion of glioma cells (Baldwin *et al.* 2010; Desai *et al.* 2011; Desai *et al.* 2012).
RAC1	1.465	RAC1 encodes Rac1, which belongs to Rho GTPase family and acts to promote cytoskeletal rearrangement and hypoxia-induced angiogenesis (Kaibuchi *et al.* 1999; Cantley 2002; Xue *et al.* 2011)	Decreased Rac1 expression was observed in astrocytic gliomas (Khalil and El-Sibai 2012). The functions of Rac1 are involved in enhancing tumourigenesis, altering cellular morphology, promoting cell migration and invasion (Salhia *et al.* 2005; Nakada *et al.* 2006; Feng *et al.* 2011). Rac1 activity is positively correlated with self-renewal, proliferation, invasion, and migration of glioma stem cells (Yoon *et al.* 2011).
RHOA	1.300	RHOA encodes RhoA GTPase which is involved in regulation of cell morphology and cytoskeletal rearrangement, such as stress fiber induction, focal adhesion, cellular division and movement, smooth muscle contraction and neurite retraction. With regard to the regulators of RhoA, guanine nucleotide exchange factors (GEFs) activator of RhoA whereas GAPs and GDP dissociation inhibitor (GDI) act	The expression level of RhoA is positively correlated with the pathological grades of astrocytomas (Yan *et al.* 2006). Although RhoA promotes cell migration in normal cells, this protein impairs cell motility in gliomas (Cachero *et al.* 1998). In addition RhoA is involved in regulation of radioresistant phenotype in glioblastoma (McLaughlin *et al.* 2006).

		as inhibitors of RhoA (Kaibuchi *et al.* 1999). Class I PI3K negatively regulates RhoA through its downstream PtdIns(3,4,5)P3/ASAP3 signaling (Krugmann *et al.* 2002).	
STAT3	1.603	STAT3 encodes Stat3 transcription factor, which regulates many cellular activities, including proliferation, differentiation, survival and inflammation. Many proteins, including RTKs, cytokine receptor, Src proteins, Jak2, mTOR and p38MAPK, were reported to regulate Stat3 in response to stimulation of growth factors, cytokinases and hormones (Yokogami *et al.* 2000; Xu *et al.* 2003; Yue and Turkson 2009).	Constitutive activation of Stat3 protein in high-grade glioblastoma in the absence of molecular alteration was reported. The functions of Stat3 in glioma and glioblastoma are promoting cell proliferation and survival, and probably involved in angiogenesis (Liu *et al.* 2010). Stat3 activation played a critical role in the maintenance of GBM stem cell properties, including proliferation, self-renewal and multipotency. In addition, Stat3 in stem cells derived from GBM was found to inhibit immune response, particularly T cell-associated activities (Liu *et al.* 2010; Sai *et al.* 2012).
Class I PI3K signaling network			
FKBP1A	1.172	FKBP1A encodes FK506 binding protein (FKBP) 1A, 12kDa (FKBP12), which belongs to FKBP protein family. FKBP12 can bind to some immunosuppressive drugs such as Rapamycin and FK506. FKBP12 also binds to ryanodine receptors (RyRs), which are intracellular calcium release channels in muscle, for enhancing RyRs stability and promoting channel gating. FKBP12 inhibits TGF-β	Up-regulation of genes encoding components of EGFR/FKBP12/HIF-2α core signaling was common in malignant childhood astrocytomas (Khatua *et al.* 2003).

		signaling through interacting with TGF-β receptor type I (Kang *et al.* 2008).	
Translation initiation factor			
EIF1	1.442	EIF1 encodes eIF1, serving as a subunit of 43S pre-initiation complex which binds to 5' capped mRNA and is required for translation initiation (Preiss and Hentze 2003).	N/A
EIF1AX	-1.714	EIF1AX encodes X chromosome-linked eIF1A, serving as a subunit of 43S pre-initiation complex which binds to 5' capped mRNA and is required for translation initiation (Preiss and Hentze 2003).	N/A
EIF2A	-1.402	EIF2A encodes eIF2A, which transfers initiator-methionyl-transfer-RNA (Met-tRNAi^{Met}) onto 40S ribosome. eIF2A is suggested to participates in initiating translation of a small subset of mRNAs (Zoll *et al.* 2002).	N/A
EIF2B1	-2.190	EIF2B1 encodes the α subunit of eIF2B, a GEF. eIF2B acts to convert inactive state of GDP bound eIF2 into active state of GTP-bound eIF2, which is a subunit of 43S pre-initiation complex (Preiss and Hentze 2003; Mohammad-Qureshi *et al.* 2008).	N/A
EIF2B2	-2.551	EIF2B2 encodes the β subunit of eIF2B. The function of eIF2B is described as aforementioned (see EIF2B1).	N/A
EIF2B3	-1.926	EIF2B3 encodes the γ subunit of eIF2B. The function of	N/A

		eIF2B is described as aforementioned (see EIF2B1).	
EIF2B4	-1.459	EIF2B4 encodes the δ subunit of eIF2B The function of eIF2B is described as aforementioned (see EIF2B1).	N/A
EIF2C1	1.763	EIF2C1 encodes eIF2C, isoform 1, which plays a critical role in RNA interference and inhibit mRNA translation (Doi *et al.* 2003).	N/A
EIF2C3	1.438	EIF2C3 encodes eIF2C3, isoform 3, which plays a critical role in RNA interference and acts to inhibit mRNA translation (Meister *et al.* 2004).	N/A
EIF2S1	-2.288	EIF2S1 encodes the α subunit of eIF2 (eIF2α), which is a heterotrimeric protein and serves as a subunit of 43S pre-initiation complex which is required for translation initiation (Preiss and Hentze 2003).	eIF2α was reported to promote the growth of malignant glioma cells (Kambara *et al.* 2005). Some drugs, such as Candidaspongiolide and Cannabioid, were reported to induce apoptosis of glioma cells *in vitro* through eIF2α-dependent ER stress or caspase12 pathway (Trisciuoglio *et al.* 2008; Salazar *et al.* 2009).
EIF2S2	-1.365	EIF2S2 encodes the β subunit of eIF2 (eIF2β). The eIF2β is responsible for GTPase activity and transfers Met-tRNAi^{Met} to the γ subunit of eIF2 (eIF2γ) initiation (Preiss and Hentze 2003).	N/A
EIF2S3	-1.363	EIF2S2 encodes eIF2γ, which interacts with both GTP and Met-tRNAi^{Met} (Preiss and Hentze 2003).	N/A
EIF3A	-1.572	EIF3A encodes the a subunit of eIF3 (eIF3a). There are 13	N/A

		subunits comprising eIF3 (Hershey 2010). eIF3 is a subunit of 43S pre-initiation complex which is required for translation initiation (Preiss and Hentze 2003). eIF3a is dispensible for eIF3-based translation initiation. eIF3a is involved in regulation of G1/S phase transition through regulation of a small number of specific mRNAs, such as $p27^{kip1}$ (Saletta *et al.* 2010).	
EIF3B	-2.253	EIF3B encodes the b subunit of eIF3. The eIF3 is a subunit of 43S pre-initiation complex which is required for translation initiation (Preiss and Hentze 2003; Hershey 2010).	It was reported that inhibition of EIF3B expression accumulated human glioblastoma cells at G0/G1 phase and induced apoptosis (Liang *et al.* 2012).
EIF3D	-1.696	EIF3D encodes the d subunit of eIF3. The eIF3 is a subunit of 43S pre-initiation complex which is required for translation initiation (Preiss and Hentze 2003; Hershey 2010).	N/A
EIF3E	-1.672	EIF3E encodes the e subunit of eIF3 (eIF3e). The eIF3e subunit was reported to arrest mRNA translation through binding to p56 interferon-induced protein (Hershey 2010).	N/A
EIF3G	-1.215	EIF3G encodes the g subunit of eIF3 (eIF3g). The eIF3g subunit promotes translation by interaction with poly(A)-binding protein-interacting protein 1 (PAIP-1) that acts to enhance translation initiation (Martineau *et al.* 2008).	N/A
EIF3H	-1.347	EIF3H encodes the h subunit of eIF3 (eIF3h). The function of eIF3h is associated with	N/A

		malignancy phenotype and acceleration of mRNA translation in cancer (Hershey 2010).	
EIF3I	-1.515	EIF3I encodes the i subunit of eIF3 (eIF3i). Like eIF3h, eIF3i is also involved in malignancy phenotype and acceleration of mRNA translation in cancer (Hershey 2010). Besides, eIF3i was reported to inhibit TGFβ signaling through interaction with type II TGFβ receptor (Choy and Derynck 1998).	N/A
EIF3J	-2.040	EIF3I encodes the j subunit of eIF3 (eIF3j). eIF3j acts to transfer eIF3 to 40S ribosome, stabilize the interaction between eIF3 and 40S ribosome, and regulate the binding of mRNA to 40S ribosome (Fraser *et al.* 2004; Fraser *et al.* 2007).	N/A
EIF4A1	-1.871	EIF4A1 encodes eIF4A isoform 1 (eIF4AI). There are three eIF4A isoforms, including eIF4AI, eIF4AII and eIF4AIII. eIF4A displays RNA-dependent ATPase activity and serves as a helicase to unwind secondary structure of 5'capped mRNA in an ATP-dependent manner. The helicase activity of eIF4A is activated by eIF4B. eIF4A is often in complex with eIF4E and eIF4G to form eIF4F complex, which is required for cap-dependent translation (Gingras *et al.* 1999; Rogers *et al.* 2002).	N/A
EIF4A3	-1.255	EIF4A3 encodes eIF4A isoform 3 (eIF4AIII). The functions of eIF4A have been described as	N/A

		aforementioned (see EIF4A1).	
PABPC1	-1.228	PABPC1 encodes PABP1, which acts to enhance the initiation step of cap-dependent translation by binding to the 3' end poly(A) tail of capped mRNA (Sachs and Varani 2000; Khaleghpour *et al.* 2001).	N/A
PAIP1	1.345	PAIP1 encodes PAIP-1, which acts to enhance initiation step of cap-dependent translation by binding to PABP-1 (Khaleghpour *et al.* 2001).	N/A
PAIP2	1.764	PAIP2 encodes PAIP-2, which acts to abrogate initiation step of cap-dependent translation by competing with PAIP1 for binding to PABP1 (Khaleghpour *et al.* 2001).	N/A
Pro-survival or anti-apoptosis			
BCL2	1.918	BCL2 encodes Bcl-2 pro-survival factor which serves as a regulator of mitochondrial change during cell death (Skommer *et al.* 2007).	Bcl-2-dependent pro-survival activity plays a key role in the resistance of glioma cells to chemotherapy and radiation therapy. In addition, Bcl-2 promotes the proliferation and progression of gliomas. However, up-regulation of Bcl-2 is more common in benign gliomas than malignant glioblastomas, presumably due to its up-regulation of $p27^{kip1}$ cell cycle inhibitor, inhibition of phosphorylated Rb and pro-apoptotic activity when binding to Nur77/TR3 protein (Kouri *et al.* 2012).
BCL2L1	1.536	BCL2L1 encodes Bcl-xL pro-survival factor, which belongs to Bcl-2 protein family. Bcl-xL serves as a regulator of mitochondrial change during	Bcl-xL was reported to promote survival and invasiveness of high-grade gliomas (Weiler *et al.* 2006).

		cell death (Skommer *et al.* 2007).	
MCL1	1.734	MCL1 encodes myeloid cell leukemia sequence 1 (Mcl-1) pro-survival factor which belongs to Bcl-2 protein family. Mcl-1 serves as a regulator of mitochondrial change during cell death (Skommer *et al.* 2007). Transcription of MCL1 gene is stimulated by class I PI3K/Akt signaling (Wang *et al.* 1999). PKC, Erk and GSK3 have been reported to be the regulators of Mcl-1 at post-translational level. The former two kinases acted as activator of Mcl-1 whereas the latter one acted as inhibitor of Mcl-1 (Domina *et al.* 2004; Maurer *et al.* 2006).	Mcl-1 was reported to maintain survival of high-grade gliomas (Sheng *et al.* 2010).
Bone morphogenetic receptor IB/Smad signaling			
BMPR1B	-1.320	BMPR1B encodes type IB BMP receptor, which belongs to TGFβ superfamily. BMP exerts its cellular effect through binding to both type I and II BMP receptors. The activated BMP receptors transduce signals to, or activate, Smad1/5/8 pathway-regulated transcription of genes. BMP signaling is involved in regulation of early embryonic development, cytoskeletal rearrangements, quiescence and angiogenesis (Sieber *et al.* 2009).	Higher expression level of type IB BMP receptor was detected in high-grade glioblastomas than that in low-grade gliomas, suggesting that this protein might contribute to malignant phenotype of gliomas (Yamada *et al.* 1996). BMP signaling pathway was reported to promote differentiation of CSCs derived from a subgroup of glioblastomas (Lee *et al.* 2008).
Wnt/beta-catennin			
CTNNB1	4.145	CTNNB1 encodes β-catenin, which serves as an important component of Wnt core	Overexpression of β-catenin and other member proteins of Wnt signaling was frequently

		signaling. Wnt/β-catenin signaling was reported to exert dual effects on stem cells. On one hand, this pathway acted to maintain self-renewal and pluripotent properties of ES cells and somatic SCs. On the other hand, Wnt//β-catenin signaling triggered differentiation and regulated lineage commitment of ES cells (Miki *et al.* 2011).	detected in GBM (Nager *et al.* 2012). Wnt/β-catenin was reported to contribute to promoting proliferation and the resistance mechanism of glioma cells to chemotherapy and radiation therapy. Besides, active β-catenin may up-regulate invasiveness and EMT in response to growth factor stimulation (Nager *et al.* 2012; Shi *et al.* 2012). Activation of Wnt/ β-catenin signaling was found to promote self-renewal and proliferation, and prevent differentiation of glioma CSCs (Nager *et al.* 2012).
MAPK pathway			
MAP2K1	1.308	MAP2K1 encodes Mek1 kinase, which serves as an important component of Raf/Mek/Erk axis pathway. RTKs and GPCRs often serve as activator of Raf/Mek/Erk pathway through Ras. This pathway is involved in regulation of transcription, proliferation, survival and cap-dependent mRNA translation (Roux and Blenis 2004). However, excessive activation of Raf/Mek/Erk signaling was reported to cause senescence-like growth inhibition in normal human fibroblasts and astrocytes. This senescence-like growth arrest was mediated by activation of either Mek1 or Raf1 (Fanton *et al.* 2001).	Active B-Raf/Mek/Erk signaling, which contributes to promoting proliferation and survival of tumour cells, is much more common in paediatric benign glioma than in paediatric malignant glioblastomas (Hargrave 2009).

MAPK1	1.461	MAPK1 encodes Erk2 kinase, which serves as an important component of Raf/Mek/Erk axis pathway. RTKs and GPCRs often serve as activator of Raf/Mek/Erk pathway through Ras. This pathway is involved in regulation of transcription, proliferation, survival and cap-dependent mRNA translation (Roux and Blenis 2004).	Activation of Erk contributes to proliferation, survival, radioresistance, invasion and migration of glioma cells (Pelloski *et al.* 2006; Song and Moon 2006; Hargrave 2009; Das *et al.* 2011).
MAPK14	-1.335	MAPK14 encodes p38 MAPK protein, which is frequently activated by cytokines and various stresses such as hypoxia and UV irradiation. P38 MAPK is involved in regulation of inflammatory and immune responses, transcription and cap-dependent mRNA translation (Roux and Blenis 2004) .	p38 MAPK exerts dual effects on glioma. On one hand, p38MAPK was reported to antagonize activities against glioma progression by enhancement of chemosensitivity and inhibition of growth and proliferation, and induction of apoptosis (Amantini *et al.* 2007; Yao *et al.* 2008; Yao *et al.* 2008; Lou *et al.* 2009). On the other hand, this protein was reported to reduce patients' survival time, promote invasion/migration, and up-regulate VEGF secretion (Song and Moon 2006; Yoshino *et al.* 2006; Demuth *et al.* 2007).
RAF1	1.659	RAF1 encodes Raf1 (also termed c-Raf) kinase, which serves as an important component of Raf/Mek/Erk axis pathway. RTKs and GPCRs often serve as activator of Raf/Mek/Erk pathway through Ras. This pathway is involved in regulation of transcription, proliferation, survival and cap-dependent mRNA translation (Roux and Blenis 2004).	The level of Raf1 expression is associated with the poor outcome of patients with malignant glioblastomas (Hagemann *et al.* 2009). Active Raf1 was reported to positively regulate invasion and tumourigenesis of gliomas. But paradoxically, Raf1 could arrest cell cycle though up-regulation of its downstream effectors, $p21^{WAF1}$ and

		However, excessive activation of Raf/Mek/Erk signaling was reported to cause senescence-like growth inhibition in normal human fibroblasts and astrocytes. This senescence-like growth arrest was mediated by activation of either Mek1 or Raf1 (Fanton *et al.* 2001).	$p16^{Ink4a}$/pRb pathways (Fanton *et al.* 2001; Lyustikman *et al.* 2008; Hagemann *et al.* 2009; Das *et al.* 2011).
RPS6KA1	-1.333	RPS6KA1 encodes RSK, isoform 1 (RSK1). There are four isoforms of RSKs, including RSK1, RSK2, RSK3 and RSK4. RSKs promote cellular proliferation and survival, and regulate transcription. Erk and PDK1 were identified to be the activators of RSKs (Roux and Blenis 2004).	N/A
RPS6KA2	-1.589	RPS6KA2 encodes RSK2. RSKs promote cellular proliferation and survival, and regulate transcription. Erk and PDK1 were identified to be the activators of RSKs (Roux and Blenis 2004).	N/A
FOS	7.450	FOS encodes c-Fos which belongs to Fos protein family. Fos family is a subunit of AP-1 heterodimer. Another subunit of AP-1 is Jun protein family. AP-1 regulates differentiation, proliferation, survival and apoptosis through up-regulating or down-regulating transcription of its target genes after AP-1 receives stimuli such as cytokines, growth factors and stress (Hess *et al.* 2004). c-Fos was reported to be activated by Ras and components of MAPK pathway, including RSK, Erk	The expression level of c-Fos was found to be positively correlated with the malignant degree of glioma (Yu *et al.* 1999; Assimakopoulou and Varakis 2001). Abberent methylation of FOS in glioma has been observed and supposed to promote glioma progession (Uyeno *et al.* 1996). c-Fos plays a role in positively regulating proliferation of glioma cells (Koul *et al.* 2007).

		and Jnk (Deng and Karin 1994; Roux and Blenis 2004) .	
JUN	1.395	JUN encodes c-Jun, which serves as a subunit of AP-1 heterodimer. AP-1 regulates differentiation, proliferation, survival and apoptosis through up-regulating or down-regulating transcription of its target genes after AP-1 receives stimuli such as cytokines, growth factors and stress (Hess *et al.* 2004). C-Jun was reported to be regulated by Jnk and RSK (Roux and Blenis 2004). The functions of c-Jun are involved in regulation of survival, apoptosis, regeneration and proliferation of cells (Herdegen *et al.* 1997; Behrens *et al.* 1999).	High expression level of phosphorylated c-Jun was commonly detected in high-grade glioblastomas but rarely in low-grade gliomas, indicating that phosphorylated c-Jun was positively correlated with malignant degree of gliomas (Assimakopoulou and Varakis 2001).
SOCS6	1.319	SOCS6 encodes SOCS6 protein, which belongs to SOCS protein family. SOCS family serves as a subunit of E3 ubiquitin ligase which degrades target proteins. In addition, SOCS family functions in inactivation of Jak/Stat pathway (Akhtar and Benveniste 2011) However, SOCS6 was reported to inhibit KIT-mediated Erk and p38 MAPK pathways, but not Akt and Stat pathways (Bayle *et al.* 2004; Zadjali *et al.* 2011).	N/A
Sodium channel			
ACCN1	2.253	ACCN1 encodes acid-sensing ion channel-2 (ASIC2) which belongs to degenerin/epithelial sodium channel (DEG/ENaC) superfamily. ASIC2 serves as a proton-gated cation channel and	N/A

		acts to sense sour in taste buds and regulates acid-dependent nociception in central and peripheral nervous system (Shimada *et al.* 2004).	
CNKSR3	1.555	CNKSR3 encodes CNKSR family member 3, which is responsible for ENaC-dependent transepithelial sodium transport and is involved in regulation of Mek activity. Expression of CNKSR3 gene is modulated by mineralocorticoid receptor in kidney in response to aldosterone stimulation (Ziera *et al.* 2009).	N/A
NEDD4	1.750	NEDD4 encodes neural precursor cell expressed, developmentally down-regulated 4 (NEDD4) protein. NEDD4 acts to inhibit the activity of sodium channel in epithelial and neural cells in response to elevated sodium concentration within cells. In addition, NEDD4 serves as an ubiquitin ligase for ubiquitylation and degradation of the targeted proteins, such as TGFβ-sepcific Smad2 (Dinudom *et al.* 1998; Goulet *et al.* 1998; Kuratomi *et al.* 2005).	N/A
Erk or Jun MAPK; AP-1 activity for proliferation, differentiation and neuronal transduction			
PRKCD	1.835	PRKCD encodes novel PKCδ serine/threonine kinase. PKCδ acts to arrest growth, promote differentiation and apoptosis, abrogate epithelial chloride secretion, inhibit Stat signaling, and activate AP-1 transcription and Mek/Erk pathway. Src and	Previous studies showed that PKCδ induced apoptosis, inhibited migration, but promoted invasion of glioma (Lu *et al.* 2007; Reyland 2007; Kim *et al.* 2008; Sarkar and Yong 2010; Ziv-Av *et al.*

		Abl were identified as activators of PKCδ (Gschwendt 1999; Tang *et al.* 2010).	2011). PKCδ played a role in expanding glioma stem cell pool and up-regulating expression levels of self-renewal-related proteins upon radiation treatment (Kim *et al.* 2011).
PRKD3	1.265	PRKD3 encodes PKD3, which is a member of PKD protein family. PKDs act to inhibit cell motility, regulate transcription, maintain oxidative stress-regulated cell survival, modulate immune responses of T and B cells, and Golgi vesicle-associated activities. Many extrcellular substances such as hormones, growth factors and neurotransmitters can activate PKDs through receptor/PLC/PtdIns(4,5)P2/diacylglycerol (DAG)/PKC pathway (Fu and Rubin 2011).	N/A
Integrin pathway			
ITGA5	1.573	ITGA5 encodes the α5 subunit of integrin receptor, which is a heterodimer composed of multiple α and β subunits. The main function of integrin receptors is to regulate the cell-cell or cell-ECM (extracellular matrix) adhesion, migration, invasion and actin-based cytoskeletal rearrangement (Miyamoto *et al.* 1995). Besides, integrin receptors are involved in regulation of cell growth, embryonic development and angiogenesis through their downstream adaptor proteins (Cox *et al.*	High expression levels of integrin receptors α5β3 and α5β5 in tumour cells and blood vessels in gliomas have been described. These integrin receptors have been reported to positively regulate adhesion, migration and angiogenesis of malignant glioblastomas and contribute to radioresistant mechanism (Tabatabai *et al.* 2010).

		2010).	
ITGB1	1.334	ITGB1 encodes β1 subunit of integrin receptor, which is a heterodimer composed of multiple α and β subunits. The main function of integrin receptors is to regulate the cell-cell or cell-ECM adhesion, migration, invasion and actin-based cytoskeletal rearrangement (Miyamoto *et al.* 1995). Besides, integrin receptors are involved in regulation of cell growth, embryonic development and angiogenesis through their downstream adaptor proteins (Cox *et al.* 2010).	Recently, integrin receptor α6β1 has found to be abundantly expressed in glioma stem cells and be involved in up-regulation of self-renewal, proliferation and tumourigenic capabilities (Lathia *et al.* 2010).
IGFBP10	1.171	IGFBP10 encodes IGFBP10 protein, also named Cyr61, which promotes cell proliferation in response to growth factor stimulation and cell adhesion through interaction with integrin receptors. Other actions exerted by Cyr61 include cartilage cell differentiation, angiogenesis, survival and apoptosis. Expression of IGFBP10 gene is predominantly activated in response to growth factors and TGF-β and is through RhoA GTPase and p38 MAPK pathways (Chen and Du 2007).	High expression level of Cyr61 was detected in gliomas (Xie *et al.* 2004). Cyr61 was reported to promote growth, invasion, migration, progression and perhaps chemoresistant mechanism of gliomas (Xie *et al.* 2004; Young and Van Brocklyn 2007; Le Mercier *et al.* 2008; Young *et al.* 2009; Goodwin *et al.* 2010).
RRAS	1.770	RRAS encodes ras-related protein (RRAS), which is a member of Ras GTPase family. RRAS acts to activate integrin receptor and regulate actin-based cytoskeletal arrangement, such as cell spreading, migration and protrusion, and	N/A

		phagocytosis, in part through its substrate phospholipase C epsilon type (PLC-ε) (Ada-Nguema *et al.* 2006).	
Serine/threonine phosphatase			
PPP1CC	-1.266	PPP1CC encodes the catalytic subunit of protein phosphatase 1 (PP1), γ isoform (PP1γ). PP1 is a heterodimer composed of a catalytic and a regulatory subunit. The catalytic subunit of PP1 acts to dephosphorylate its target kinases from serine/threonine residues. PP1 is involved in regulation of various cellular processes, such as glycogen synthesis, cytoskeletal organization and cell proliferation, by its catalytic subunit-dependent dephosphorylation of its target proteins and the location of its regulatory subunit (Cohen 2002).	PP1 was report to promote progression of glioma by degradation of glycogen into glucose under glucose deprivation condition (Kelsall *et al.* 2011).
PPP1R10	-1.337	PPP1R10 encodes the regulatory subunit 10 of PP1, which is distributed in nucleus of all cell types. PP1 that contains the regulatory subunit 10 exerts its action in specific RNA-binding (Cohen 2002; Kim *et al.* 2003).	N/A
PPP1R12A	-1.398	PPP1R12A encodes the regulatory subunit 12A of PP1, which is distributed in smooth muscle and regulates smooth muscle relaxation through dephosphorylation of myosin (Cohen 2002).	N/A
PPP1R14B	-2.333	PPP1R14B encodes the regulatory subunit 14B of PP1, which is ubiquitously expressed	N/A

		and acts to inhibit PP1 activity (Cohen 2002).	
PPP1R3C	7.969	PPP1R3C encodes the 3C regulatory subunit of PP1, which is ubiquitously expressed in all types of cells, particularly in liver and muscle cells and functions in promoting glycogen synthesis (Cohen 2002).	N/A
PPP1R7	-1.575	PPP1R7 encodes the regulatory subunit 7 of PP1, which is ubiquitously expressed in nucleus of all types of cells and promotes cell progression from metaphase to anaphase (Cohen 2002).	N/A
PPP2CB	1.235	PPP2CB encodes protein phosphatase 2, catalytic subunit, beta isoform (PP2Ac-β), which is widely distributed, particularly in brain and heart. PP2A acts to arrest G2/M phase transition, and inhibit cap-dependent translation and signal transductions, promote apoptosis through dephosphorylation of protein kinases (such as mTOR, Erk and caspase-3) on serine/threonine residues (Janssens and Goris 2001).	Evidence suggested that PP2A might induce differentiation and/or death of GSCs through decreasing Akt phosphorylation and increasing the expression level of nuclear receptor corepressor (N-CoR) (Lu *et al.* 2010).
PPP2R3B	-1.583	PPP2R3B encodes protein phosphatase 2, regulatory subunit B", β isoform (also named PR48). PR48 is distributed in nucleus in mammalian cells and acts to arrest G1/S transition (Yan *et al.* 2000).	Evidence suggested that PP2A played a role in decreasing Akt phosphorylation and increasing the expression level of N-CoR which is overexpressed in CSCs derived from GBM (Lu *et al.* 2010). In addition, PP2A or PP1 might be involved in regulation of ethanol-inhibited the import of adenosine into

			glioma cells (Coe *et al.* 1996).
PPM1J	-1.418	PPM1J encodes protein phosphatase 2C, zeta isoform (PP2C-ζ), which is a member of PP2C serine/threonine protein phosphatase family. PPM1J is involved in degradation of proteins through binding to ubiquitin conjugating enzyme 9 (Kashiwaba *et al.* 2003).	N/A
Cytoskeletal rearrangement for cell migration, cell division and intracellular trafficking			
FNBP1	1.874	FNBP1 encodes formin binding protein 17 (FBP17) protein, which is a member of CIP4 subfamily of F-BAR protein family. FBP17 is the binding partner of three fomin proteins, which are mDia1, mDia2 and DAAM1. FBP17 is required for regulation of formin-based actin polymerization and is involved in many cellular processes, such as motility, division and endocytosis/phagocytosis (Aspenstrom 2010).	N/A
RHOC	1.477	RHOC encodes ras homolog gene family (Rho), member C (RhoC), which is a member of Rho GTPase protein family. RhoC is widely distributed in cytosol and acts to promote cell motility (Wennerberg and Der 2004; Wheeler and Ridley 2004).	A study suggested that RhoC was involved in regulation of microRNA-10b-induced invasion of glioma (Sasayama *et al.* 2009).
RHOJ	-1.559	RHOJ encodes Rho, member J (RhoJ), which is a member of Rho GTPase family. RhoJ mediates migration, tubule generation and actomyosin contractility of endothelial cells in response to VEGF	N/A

		stimulation, clathrin-dependent endocytosis, and adipocyte differentiation in response to peroxisome proliferator-activated receptor γ (PPARγ) stimulation (de Toledo *et al.* 2003; Nishizuka *et al.* 2003; Kaur *et al.* 2011).	
RHOQ	2.132	RHOQ encodes Rho, member Q (RhoQ, also named TC10), which is a member of Rho GTPase family. TC10 is involved in actin-based cytoskeletal arrangement and GLUT4 transportation in adipocytes in response to insulin stimulation. TC10 also plays a role in regulation of neural development and plasticity (Chiang *et al.* 2002; Wennerberg and Der 2004).	N/A
RHOT1	1.540	RHOT1 encodes Rho, member T1 (RhoT1, also named Miro-1), an atypical Rho GTPase. Miro-1 is involved in apoptosis induction and mitochondrial homeostasis and trafficking (Fransson *et al.* 2003; Fransson *et al.* 2006).	N/A
RND3	1.863	RND3 encodes Rho family GTPase 3 (Rnd3), also termed RhoE. Rnd3/RhoE was reported to inhibit RhoA-modulated cellular events such as motility, focal adhesion and cell morphology, and down-regulate cyclinD1-dependent cell cycle progression, and act as a tumour suppressor (Riento *et al.* 2005).	RhoE was found to act as tumour suppressor in glioblastoma by disruption of cytoskeletal arrangement, apoptosis induction, and growth arrest (through RhoE/Erk/cycln D1/Rb pathway) (Poch *et al.* 2007).
SRF	-1.219	SRF encodes serum response factor (SRF) transcription factor, which regulates several cellular functions including	SRF was found to regulate migration of glioma, in part, by binding to RTVP-1 promoter

		contractility, motility, survival, synaptic activity and immune response through transcription of its target genes. There are two pathways which regulate SRF transcription activity, which are RTK/MAPK/ELK1 and Rho pathways (Miano 2010).	(Ziv-Av *et al.* 2011).
TRIP10	1.703	TRIP10 encodes thyroid hormone receptor interactor 10 (Trip10). The functions of Trip10 are involved in translocation of GLUT4 glucose transporter, endocytosis, cytoskeletal rearrangement, cell cycle progression and survival, all of which is cell-specific (Hsu *et al.* 2011).	N/A
Insulin-related glucose uptake			
STX4	1.974	STX4 encodes syntaxin 4. The syntaxin 4 promotes exocytosis or secretion of insulin from pancreatic β islet cells and insulin-induced translocation of the glucose transporter GLUT4 which is attached on vesicles from cytosol to cell surface, which allows glucose uptake in skeletal muscle cells and adipocytes (Jewell *et al.* 2010).	N/A
STXBP4	-1.855	STXBP4 encodes synip, which functions as a negative regulator of syntaxin4 through binding to syntaxin 4. The insulin/class I PI3K/Akt-mediated phosphorylation of synip results in dissociation of synip from syntaxin 4. Subsequently, the free syntaxin can recruit the glucose transporter GLUT4 attached on vesicles from cytosol to cell	N/A

		surface, which allows glucose uptake in adipocytes (Yamada *et al.* 2005).	
TGFBR2	-1.495	TGFBR2 encodes TGFβ receptor type II (TβRII). The TGFβ-RII activates its downstream Smad2/3 signaling through phosphorylation of TGFβ receptor type I (TβRI). The TGFβ signaling functions in inhibition of G1/S phase transition and promotion of EMT for cell migration. The TGFβ signaling can either promote or inhibit tumour progression, depending on tumour types (Hata 2001).	TGFβ signaling positively regulates glioma invasiveness and metastasis through up-regulation of matrix metalloprotease (MMP) expression and EMT. Besides, TGFβ signaling down-regulates MHC class II expression in immunocytes, endothelial cells and tumour cells within glioma. This inhibits the migration of immunocytes to glioma, which allows glioma to escape immune response. TGFβ signaling generally acted to arrest the growth of glioma cells *in vivo* (Wick *et al.* 2006). TGFβ signaling was reported to maintain stemness and tumour-initiating ability of glioma stem cells through up-regulating transcription of SOX4 and LIF genes (Miyazono *et al.* 2012).
14-3-3 protein family			
YWHAB	-1.223	YWHAB encodes 14-3-3β, which belongs to 14-3-3 phosphoserine/phosphothreonine-binding protein family. Over 100 proteins, such as transcription factors, cytoskeletal components and proteins involved in signaling pathways, have been identified as the binding partners of 14-3-3 protein family. The functions of 14-3-3 proteins are involved in regulating transportation,	Increased expression of 14-3-3β protein was detected in human gliomas as compared with the surrounding normal tissue (Liang *et al.* 2009). Up-regulation of 14-3-3β gene and its protein level was positively correlated with malignancy degree of astrocytomas (Cao *et al.* 2008; Yang *et al.* 2009). 14-3-3 family proteins are

		stabilization, interaction and phosphorylated state of their protein substrates. 14-3-3β positively regulates Raf/Erk pathway through interaction with Ras. 14-3-3β induces cellular motility, spreading and integrin-associated signaling through interaction with integrins (Wilker and Yaffe 2004).	involved in maintaining survival and proliferation of xenograft of human glioma cell lines in mouse model (Cao *et al.* 2010).
YWHAG	-3.020	YWHAG encodes 14-3-3γ, which belongs to 14-3-3 phosphoserine/phosphothreonine-binding protein family (Wilker and Yaffe 2004). The general functions of 14-3-3 protein family were described as aformationed (in YWHAB).	14-3-3 family proteins are involved in maintaining survival and proliferation of xenograft of human glioma cell lines in mouse model (Cao *et al.* 2010).
YWHAQ	-1.255	YWHAQ encodes 14-3-3θ, which belongs to 14-3-3 phosphoserine/phosphothreonine-binding protein family (Wilker and Yaffe 2004). The general functions of 14-3-3 protein family were described as aformationed (in YWHAB).	The immunohistochemical intensity of 14-3-3θ protein was positively correlated with malignancy degree of gliomas(Cao *et al.* 2008). 14-3-3 family proteins are involved in maintaining survival and proliferation of xenograft of human glioma cell lines in mouse model (Cao *et al.* 2010).

N/A represents information not available.

Publication

Chen et al. BMC Veterinary Research 2012, 8:73
http://www.biomedcentral.com/1746-6148/8/73

BMC Veterinary Research

RESEARCH ARTICLE Open Access

The class I PI3K/Akt pathway is critical for cancer cell survival in dogs and offers an opportunity for therapeutic intervention

Yu-Ting Chen, Karen AL Tan, Lisa Y Pang and David J Argyle*

Abstract

Background: Using novel small-molecular inhibitors, we explored the feasibility of the class I PI3K/Akt/mTORC1 signaling pathway as a therapeutic target in canine oncology either by using pathway inhibitors alone, in combination or combined with conventional chemotherapeutic drugs *in vitro*.

Results: We demonstrate that growth and survival of the cell lines tested are predominantly dependent on class I PI3K/Akt signaling rather than mTORC1 signaling. In addition, the newly developed inhibitors ZSTK474 and KP372-1 which selectively target pan-class I PI3K and Akt, respectively, and Rapamycin which has been well-established as highly specific mTOR inhibitor, decrease viability of canine cancer cell lines. All inhibitors demonstrated inhibition of phosphorylation of pathway members. Annexin V staining demonstrated that KP372-1 is a potent inducer of apoptosis whereas ZSTK474 and Rapamycin are weaker inducers of apoptosis. Simultaneous inhibition of class I PI3K and mTORC1 by ZSTK474 combined with Rapamycin additively or synergistically reduced cell viability whereas responses to the PI3K pathway inhibitors in combination with conventional drug Doxorubicin were cell line-dependent.

Conclusion: This study highlighted the importance of class I PI3K/Akt axis signaling in canine tumour cells and identifies it as a promising therapeutic target.

Keywords: Canine, Cancer, PI3, AKT, MTOR, Therapeutic, Target

Background

The class I phosphatidylinositol 3-kinase (PI3K) signaling pathway comprises a series of serine/threonine kinase cascades that regulate a variety of cellular processes including cell cycle progression, cell survival and migration, and protein synthesis. Recent evidence supports the hypothesis that the dysregulation of class I PI3K signaling promotes tumourigenesis and angiogenesis in various cancer types [1-3].

Class I PI3K is predominantly activated by receptor tyrosine kinases (RTKs) upon receiving growth factor stimulation. The activated RTKs undergo either autophosphorylation of tyrosine (Y) residues at the intracellular domains or phosphorylation of their substrates such as IRS-1, IRS-2 and Gab on Y residues. The phosphorylated Y residues are soon recognized by SH2 domains in p85 regulatory subunit of class I PI3K, recruiting class I PI3K to plasma membrane, triggering activation of PI3K downstream pathways (reviewed in ref. [4,5]). Alternatively, class I PI3Ks can be activated through the interaction between p110 catalytic subunit and Ras following RTK activation [6-8]. The activated class I PI3K can convert phosphatidylinositol-4,5–biphosphate (PIP2) to phosphatidylinositol-3,4,5–triphosphate (PIP3), resulting in the recruitment of Akt to the plasma membrane and allowing phosphatidylinositol 3-dependent kinase 1 (PDK1) to phosphorylate and activate Akt. In contrast, Akt activity can be counteracted by phosphatase and tensin homolog (PTEN) tumour suppressor through conversion of PIP3 back to PIP2 (reviewed in ref. [9]).

The class I PI3K effects cellular functions through its two major downstream effectors Akt and mTOR. Akt can phosphorylate FoxO3a, BAX, BAD, and caspase 9 to antagonize apoptotic activity, [10-13] phosphorylate pro-survival factors such as MDM2 and IKK-α to maintain cell survival, [14,15] phosphorylate mitochondrial hexokinase-II to prevent mitochondria from initiation of apoptosis,

*Correspondence: David.argyle@ed.ac.uk
Royal (Dick) School of Veterinary Studies and the Roslin Institute, University of Edinburgh, Edinburgh, UK EH25 9RG